PATHOGENESIS OF CONGENITAL CYTOMEGALOVIRUS INFECTION: FINDING PROGNOSTIC MARKERS AND CORRELATES OF PROTECTION

ROBERTA ROVITO

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PATHOGENESIS OF CONGENITAL CYTOMEGALOVIRUS INFECTION: FINDING PROGNOSTIC MARKERS AND CORRELATES OF PROTECTION

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To all children

Look for happiness, everyday, continuously.
We always have to think about happiness,
And even if sometimes it forgets about us,
We must not ever forget about it,
Till the last day of our life!

Roberto Benigni, 2014

CHAPTER 1

GENERAL INTRODUCTION

1.1. CYTOMEGALOVIRUS INFECTION: FROM GLOBAL INFECTION TO NEGLECTED PUBLIC HEALTH CONCERN

Cytomegalovirus (CMV) infection is without a doubt a global and endemic infection. In the USA, Australia and Europe the CMV seroprevalence varies between 36% and 77%, while in developing countries this percentage often reaches 100% (Fig. 1) (1). In countries with low to moderate seroprevalence, age is a predictor of seropositivity since the chance of exposure to CMV continues throughout life. As a rule of thumb, the socioeconomic status may be a reflection of factors that contribute to the exposure to CMV, such as crowded living conditions or numerous infants. Although in a considerable proportion of immunocompetent individuals CMV infection is subclinical, it can still cause a variety of clinical manifestation among which the mononucleosis like syndrome in young individuals is the best characterized (2). CMV has also been suggested as a co-factor in the pathogenesis of inflammatory, autoimmune and vascular disease, such as atherosclerotic coronary artery disease (3-6), as well as a risk-factor for all-cause mortality in large population-based cohorts in the USA and Europe (7, 8). However, the severe morbidity caused by CMV in immunocompromised individuals and neonates, and the following long-term disability in children, accounts for the true burden of disease of CMV infections.

Despite the considerable knowledge of the contribution of CMV to mortality and morbidity in immunocompromised individuals, the disease burden of congenital CMV infection (cCMV) is less often acknowledged (9). One of the reasons of this discrepancy may lie in the epidemiology of congenital infection, which altered the dogma of pre-existing immunity to CMV in pregnant women protecting against vertical transmission. Indeed, the rate of cCMV is higher in highly seropositive countries, i.e. developing countries, where CMV is acquired very early in life. At 3 months of age two third of the infants are estimated to be infected, and 85% by the first year of life (10, 11). Here, fewer women of child-bearing age are seronegative compared to developed countries, and the rate of cCMV ranges from 1% to 5%, or even higher (11-13). In developed countries, ~50% of child-bearing age women is seronegative with a lower rate of cCMV, between 0.6% and 0.7% of live births (14-17) (Fig. 2). This discrepancy is most likely due to the fact that the seroprevalence reflects the size for the viral reservoirs in a population, though the characteristics of CMV infection make the modes of transmission and acquisition difficult to determine (see "The ancient virus" paragraph) (9). In general, when a seronegative mother gets infected during pregnancy the vertical transmission rate is 30-35%, whereas in seropositive mothers this rate is estimated to be 1.2% (18-20). Although recent observations highlighted the fact that this may be higher due to re-infections with a new viral strain (21). However, the main contributor to the worldwide number of cCMV cases is represented by seropositive mothers.

Among congenitally infected children, 12.7% are estimated to be symptomatic at birth, with the most common symptoms being petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly. An estimated 40-58% of these symptomatic children will develop permanent sequelae, such as hearing loss, mental retardation, and developmental delay. Importantly, of the 87.3% neonates that are asymptomatic at birth, ~13.5% will also develop permanent sequelae (16, 17) (Fig. 3). A similar frequency of symptomatic congenital infections has

been shown in mothers with primary infection and in mothers with secondary infection (being either reactivation or reinfection) (12, 22, 23), as well as similar severity of symptoms (12, 24, 25). Overall, a quite high percentage of congenitally infected neonates, estimated to be 17%, will have permanent sequelae. The majority of children with permanent sequelae come from the group that

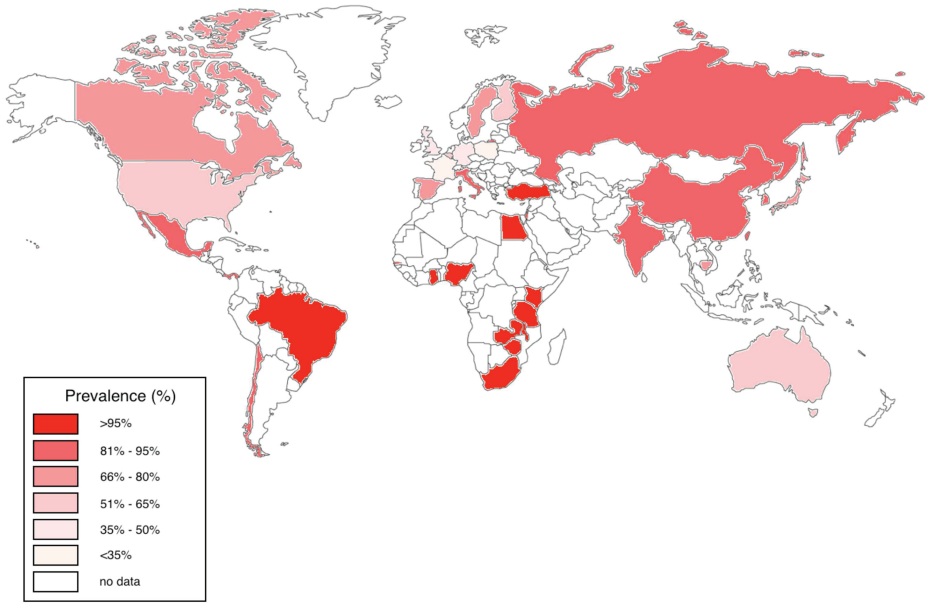

Figure 1. Worldwide CMV seroprevalence rates in adults. Reprinted with permission from (1).

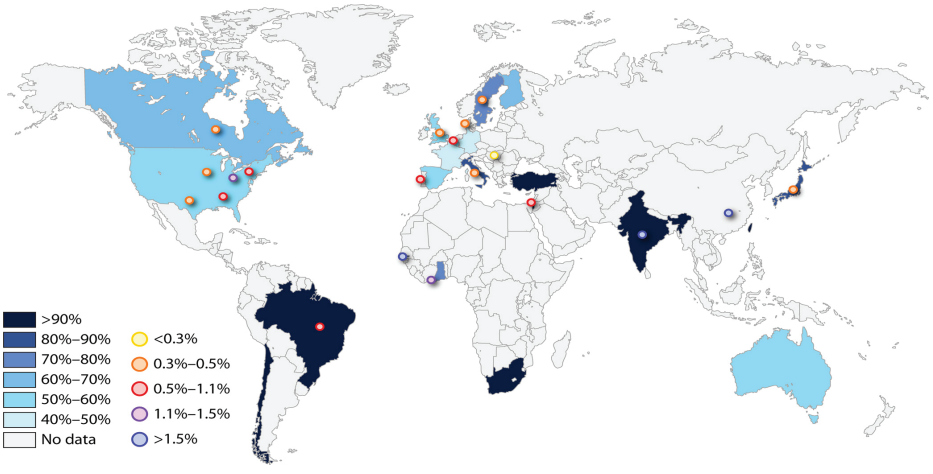

Figure 2. Worldwide CMV seroprevalence rates among women of reproductive age, and birth prevalence of congenital CMV infection. Shades represent CMV seroprevalence in women of child-bearing age, circles represent congenital CMV birth prevalence. Reprinted with permission from (9).

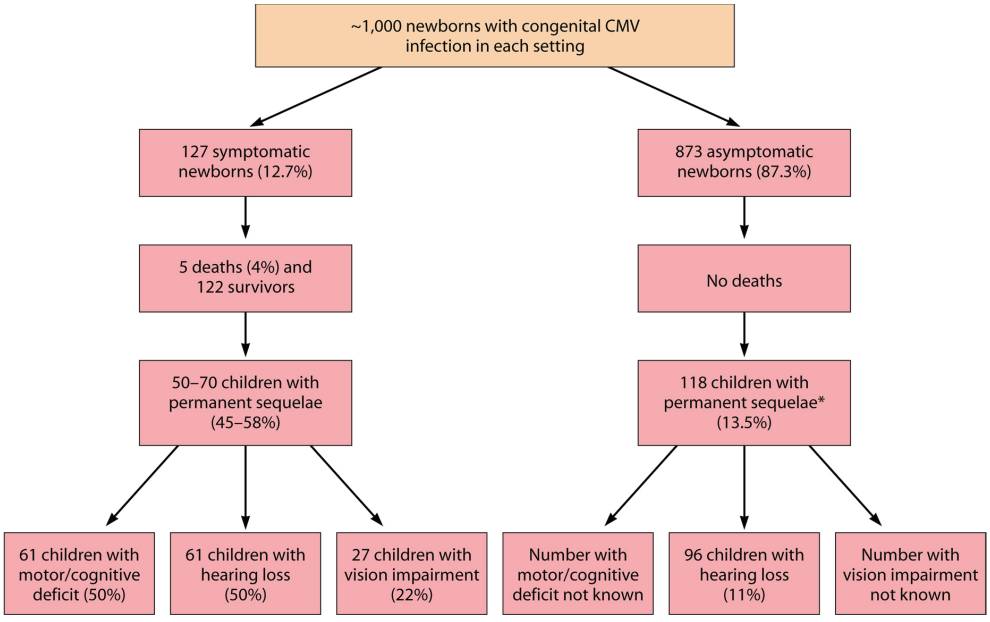

Figure 3. Estimates of the prevalence of congenital CMV infection and sequelae. Reprinted with permission from (9).

is asymptomatic at birth. In this group there are no clinical signs that would point towards a CMV infection in the neonate, therefore the infection may easily remain unnoticed, and the late onset of sequelae makes a retrospective diagnosis rather challenging.

To conclude, CMV is the most common cause of congenital infection worldwide, a much less exotic infection than newly emerging infections such as Zika virus infections, but overall a much greater global problem (16, 17).

1.2. THE ANCIENT VIRUS

Cytomegalovirus (Fig. 4) is the largest and structurally most complex member of the family of human *herpesviridae*, which includes several DNA viruses whose hallmark is latency. The pathogenic *herpesviridae* for humans are divided into three subfamilies based on structural and replicative properties. CMV belongs to the sub-family of β-*herpesvirinae*, which share a common long replicative cycle (~36-48 hours in permissive cells). The common ancestor of α-, β- and γ-*herpesvirinae* is believed to date 400 million years ago (Mya) while the β-*herpesvirinae* made their appearance only 50 Mya thereafter (350 Mya) (26). Importantly, the typical large inclusion-bearing cells produced by CMV were first reported in 1881 in a kidney of a stillborn infant with congenital syphilis (27), considerably later than their first appearance. CMV is 200 nm of diameter and has four distinct morphologic units: the *core* which contains 230 kilobase pairs of linear double-stranded DNA; the proteic *nucleocapsid* which consists of 162 capsomers (28); an amorphous layer called *tegument*

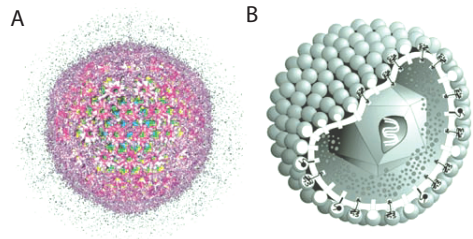

Figure 4. Human Cytomegalovirus. A) Three-dimensional reconstruction of icosahedrally portion of human CMV. B) virtual three-dimensional model showing the various CMV components. Reprinted with permission from (47).

which contains several viral proteins and RNA (29), and a double-stranded lipidic layer called *envelope*, where viral glycoproteins are embedded. The massive size of the genome is accompanied by a complex genomic organization and post-transcriptional modifications. The tegument is the most heterogeneous structure in CMV, and exerts several functions for viral replication, e.g. blocking of the cellular response that degrades entering DNA, and enhancing IE (immediate early) gene transcription (30-32). It also contains the immunodominant target of T cells and Abs, pp65 (33-36). Several glycoproteins have been described, and the pentameric complex gH/gL/gUL128-131 has been shown to elicit neutralizing Abs against several CMV strains (37-40). The initial binding between CMV and the target cell occurs via viral glycoproteins and cellular proteoglycans, followed by binding with more specific receptors and fusion/endocytosis (41-43). CMV has a broad tropism as it can be detected in fibroblasts, endothelial cells, epithelial cells, monocytes/macrophages, smooth muscle cells, stromal cells, neuronal cells, neutrophils, and hepatocytes (44-46). This suggests that the cellular receptors may be multiple, even though the primary targets are epithelial cells and monocytes. After these first events, the nucleocapsid is transported to the nucleus where the IE genes are expressed. IE1 (or pp72) is a phosphoprotein used as a target for detection of CMV infected cells, while IE2 is a transactivating protein responsible for activation of other viral genes (24). Next, the early, or β-genes, are expressed, which mainly contain viral proteins necessary for the viral genome synthesis, such as the viral DNA polymerase (24). Finally the late, or γ-genes, are expressed and these mainly encode structural proteins. The viral DNA is produced in the nucleus as concatemers, and during the packaging is cleaved and incorporated into the nucleocapsid. Though the mechanism is not completely understood, the nucleocapsid leaves the nucleus and reaches the cytoplasm as a partially tegumented viral particle. The final maturation occurs in a specialized cytoplasmatic compartment called *assembly compartment* (100). The virus egression is believed to occur via cell lysis in certain cell subtypes, e.g. fibroblasts, or via a non-completely understood mechanism of exocytosis. *Latency* is accomplished through viral genome circularization in the cellular nucleus, forming a so-called *episome* (108), and is mainly established in monocytes, CD34+ progenitors and endothelial cells. Consequently, in the host there might be a low level of productive infection with recurrent viral secretion, and several pro-inflammatory signals can induce such reactivation (106-109). This finding distinguishes acute from chronic infection. Acute infection

is characterized by an intense viral replication and shedding that lasts months, while the chronic infection is characterized by alternating replications and concomitant shedding.

1.3. TRANSMISSION OF CYTOMEGALOVIRUS

By definition the infectivity of CMV is much lower than of other viruses that have an airborne transmission (48). Therefore a close contact between a susceptible person with infected secretions is necessary for an efficient transmission. The transmission can occur via direct person-to-person or indirect contact, and sources of virus include oropharyngeal secretions, cervical and vaginal excretions, semen, breast milk, tears, urine, feces, and blood (49). CMV can therefore be transmitted horizontally through saliva, sexual contact, transplantation or breast-feeding, and vertically through the placenta. Despite the necessity of a close contact, the high seroprevalence in several countries suggests that CMV keeps spreading within a population, causing frequent infections. One of the most important contributing factors may be the high and chronic viral shedding of certain subgroups, such as congenitally and perinatally infected children, that can shed the virus for years, whereas in children and adults with primary infection this lasts ~6 months (50, 51). In addition, CMV can irregularly, and unpredictably, contribute to this frequent infection via secondary infections. In developed countries, where ~50% of women of child bearing age are seronegative, the most important source of infection is young children, possibly through their urine and saliva (52). Susceptible children are mainly infected through breast-feeding or other contacts with infected mothers and children, and only a small percentage via contact with the maternal cervical excretion during delivery (53). If infection occurs through breast-feeding, the presence of CMV in breast milk tends to be a bit later than immediately after birth, and CMV becomes detectable in the child only 6-8 weeks after the first exposure to CMV in the breast milk (54-56). Approximately 37-59% of infants breast-fed by a seropositive mother are eventually infected (54, 57).

1.4. CMV IMMUNE RESPONSE

The clinical impact of cCMV is the result of the interplay between virus and host in a stepwise process that starts in the mother, reaches the placenta and the fetus to then continue in the children throughout their childhood. In the following paragraphs these compartments will be discussed separately, although the processes within *one mother-child pair* are obviously non-separable. The general CMV immune response will be discussed first as it helps understanding the changes occurring during pregnancy, and in the fetus.

1.4.1. General CMV immune response

Following binding of CMV to the target cell, an alteration of the host gene expression occurs (58-60), with a rapid activation of the innate defence, possibly through TLR-2 and gB, that leads to a pro-inflammatory environment, co-stimulatory molecules, DC maturation, and IFN-α/β production (59, 61). The primary aim of such a response is to control the infection, while modulating the adaptive immune response. After an initial local replication, CMV spread and dissemination is most likely cell-associated, occurring mainly through monocyte/macrophages. When infected

circulating monocytes enter host tissues, they differentiate into macrophages that can sustain viral r eplication (62).

Upon acute primary CMV infection, a high pool of CD8+ T cells with a broad non-selective repertoire against several CMV Ags is produced. In healthy individuals, CMV DNAemia has a peak at 2-3 weeks after primary infection, and it decreases in 4-5 weeks after the primary infection (63). Cytotoxic T cells, with polyclonal TCRs, appear 2-3 weeks post-infection, and at ~5-8 weeks post-infection only a few sub-populations will reach the memory pool (63). This time approximately coincides with the resolution of primary infection. The memory compartment produced upon CMV infection is much bigger than that produced in response to other viruses, and it increases with age reaching 5% of all peripheral CD8+ T cells for a single CMV epitope (64). This phenomenon is called *memory inflation* and it may be attributable to the intermittent virus reactivation, and/or reinfection, that boost the T cell response.

Unfortunately, data on the CD4+ T cell response during primary CMV infection in immunocompetent individuals is not available as the majority of studies focused on latent infection. In latency the frequency of CD4+ T cells specific for CMV in healthy donors was 1-2% of all peripheral CD4+ T cells (65). However, some important conclusions drawn from transplantation studies may apply to the general population; though one should keep in mind that the immunosuppressive therapy might slightly change the kinetics of infection. In seronegative individuals that received a renal transplant from seropositive donors, viremia was detected ~25 days after transplantation, and CD4+ T cells appeared ~7 days after the first detection of viremia to then reach ~2.5% of the total of peripheral CD4+ T cells and again decrease to low level in the following ~2-3 months (65). Additionally, even though the detection of CMV DNA was similar, the peak viral load and the duration of viremia was higher and longer in those who had symptoms (66). Interestingly, in immunocompetent children an impaired CD4 response was associated with longer viral shedding in both urine and saliva (67).

Although it is commonly recognised that Abs have an important role during CMV infection, the majority of studies focused on the T cell response, and very little is known on the B cell compartment, and its kinetics during CMV infection. The protective effect of high Abs titers has been shown in several target populations (68-72). E.g. in immunocompetent individuals, a primary CMV infection induced an early and remarkable Abs response regardless of the clinical outcome, however, in those individuals with a more severe manifestation a deficient neutralizing Ab response was observed (69). Unfortunately, the real mechanism through which these Abs act in vivo still needs to be elucidated.

This remarkable CMV-specific immune response has an important feature, which is T and B cell *exhaustion*, though most studies focused on T cell exhaustion (73). T and B cell exhaustion is a state of dysfunction induced by chronic infections and prolonged exposure to high viral loads (73-75), and it is characterised by poor effector functions, expression of inhibitory molecules and specific transcriptional state (76). However, a certain degree of reversibility of this state has been shown through e.g. block of PD-1 (77). Importantly, exhaustion is a different concept than *anergy* or senescence. *Anergy* is considered to be a state of non-responsiveness that is initiated rapidly upon

first encounter with the Ag. Whereas, *senescence* is considered to be the terminal differentiation of a cell that loses the proliferative potential, and is typical of a latent infection.

1.4.2. Maternal CMV immune response

During pregnancy, the need to maintain the balance between fetal tolerance and antiviral immunity complicates the aforementioned immune response to CMV. Pregnancy does not seem to considerably alter the CMV-specific cell-mediated immune response compared to that of immunocompetent non-pregnant women (78, 79). However, in pregnant women who transmitted CMV to their fetus (transmitter), the lymphoproliferative response to CMV was lower and delayed (78, 79). Additionally, the duration of viremia was similar between transmitter and non-transmitter women (79). For what concerns the humoral response, Abs against gB were found to be higher during delivery in transmitter mothers, though the level of neutralizing Abs was lower (68). Additionally, primary CMV infection increased the expansion of activated and atypical memory B cells, enriched for CMV-specific cells, compared to the chronic infection. In the latter, the frequencies were similar to those of healthy adults, suggesting that the expansion does not last long (73), though the presence of memory B cells ensures a quick Abs production upon reactivation/reinfection.

1.4.3. Placental CMV immune response

The maternal immune system at the decidua level is mainly characterized by decidual natural killer cells (dNK), around 10% of leukocytes are T cells, both αβ and γδ subsets, while the frequency of B cells is low (80-82). At the maternal-fetal interface the majority of trophoblast cells are in direct contact with maternal cells, and fetus-specific maternal immune cells have been shown both locally and peripherally (83, 84). In normal conditions it does not damage pregnancy as several mechanisms are in place to prevent rejection of the fetal semi-allograft. Extravillous trophoblast cells do not express HLA-A, -B, -DR, -DQ and –DP (85), but they do express HLA-C and the non-classical HLA-E and HLA–G. HLA-C and HLA-E prevent maternal NK cell-mediated cytotoxicity through binding with killer cell immunoglobulin-like receptors (KIRs) expressed on dNK. Whereas, HLA-G modulates the response of different cellular subsets including dNK, antigen-presenting cell (APC), T cells, and B cells (86, 87). The complex local maternal-fetal immune cross-talk differs from the peripheral immune system of both mother and child (88). Indeed, immune cells can be generated locally with a different function than the one acquired at the periphery. For example, CD8+ T cells express significantly lower levels of perforin and granzyme-B, dendritic cell (DC) are arrested in a tolerogenic state, and dNK cells can be generated locally (88, 89). Viral infections may increase the levels of pro-inflammatory cytokines, chemokines, and the influx of T cells in decidual tissues (90, 91). In this situation, the regulatory mechanisms might not be able to efficiently inhibit the allogeneic lymphocytes (89), which could damage the placenta. CMV replication occurs in the decidua, endothelial cells and endovascular cytotrophoblast (92-94). Even though maternal macrophages and NK cells may limit the viral replication in the uterine wall (93), once CMV infects the cytotrophoblasts there is an extensive change in molecule expression that hampers their migration and invasion (92, 95-98). Women with primary CMV infection and a symptomatic fetus had

thicker placentas than women with asymptomatic foetuses; additionally the latter placentas were thicker than those of women with secondary infection (99). Furthermore, the infected placentas are characterized by an hypoxic-like environment that attempts to induce compensatory mechanism by increasing the area of the fetal part of the placenta in order to increase the oxygen influx, and this may contribute to the thickening as well (100, 101). However, more data are needed on the impact of cCMV on the placental immune cross-talk and on the decidual tissues in relation to outcome.

1.4.4. Fetal and neonatal CMV immune response

How CMV reaches the fetus from the placenta is largely unknown, but maternal Abs affinity probably plays a role in the chance of fetal infection. In case of high avidity IgG, viral replication in the cytotrophoblast is limited and therefore the virus spreads less to the other layers of the placenta (93, 102-104); with low avidity IgG the viral infection is not prevented and therefore viral transport is facilitated. The placental alterations mentioned in the previous paragraph clearly induce a placental dysfunction that could hamper fetal development, and in turn contribute to the development of symptoms at birth. Upon fetal infection, the induction of a CMV-specific immune response has been shown. The majority of available data refer to αβ T cells. In congenitally infected children, there is a strong response of CD8+ T cells (10, 67, 105-110), during which they acquire a late differentiation phenotype (10, 105, 106, 111), and a restricted number of clones is generated (105). CMV-specific CD8+ T cells have been detected as early as 22-28 weeks of gestation (105, 108). During the first year of life, the number of CMV-specific IFNγ-producing CD8+ T cells increases, as well as the repertoire of CMV peptides against which they react (10, 110). Expansion of CD4+ T cells, as well as of γδT cells (112), has also been described in the context of cCMV (113). γδT cells with antiviral activity have been detected as early as 21 weeks of gestation (112). These might have a more important role in early life as they develop earlier than αβ T cells (114). Despite the oligoclonal expansion of fetal CD4+ and CD8+ T cells, similar to that in adults, their functionality and cytokines response was lower, and they showed a typical exhaustion phenotype (74). This is similar to what happens in adults after primary or chronic infection, but the magnitude of this phenomenon is more intense during fetal life (114), most likely because of the higher viral loads the foetuses are exposed to (63). This exhaustion of T cells in the foetuses may be responsible for the prolonged CMV viral excretion in these children which can last up to 5 years (50, 74). In general, also after postnatal CMV infection the viral excretion is longer, ~2 years, than in immunocompetent adults, and this suggests a limited control of the infection in early life (50, 51, 67, 114). Unfortunately, little information is available on fetal B cell immunity during cCMV. IgM positive B cells have shown to emerge in the peripheral circulation as early as 12 weeks of gestation (115), and CMV infected foetuses can produce IgM (116-118), but the antiviral activity and the role in CMV disease control have not been evaluated yet (119). The evidence of NK cell activation in the context of cCMV is scarce. An expansion of NKG2C+ NK cells was observed in infants with cCMV, and was particularly marked in symptomatic cases (120).

1.5. PROGNOSTIC MARKERS FOR cCMV CLINICAL OUTCOME

One of the main problem with cCMV is that, despite the extensive knowledge on the clinical outcome and despite being the most common congenital infection leading to a variety of permanent disabilities, the question whether a child will be symptomatic at birth or will develop LTI remains largely unanswered. This is just because cCMV pathogenesis is largely unknown. A prognostic marker for clinical outcome would help identifying subgroups of patients that would benefit from certain clinical interventions, as well as giving more insights into cCMV pathogenesis.

In the following paragraphs, the available prognostic markers are classified into different subgroups according to the main goals of prediction: vertical transmission of CMV, symptoms at birth and LTI development, and whether these measurements were performed in the mother, in the fetus or in the neonate. Whereas, the first paragraph is focused on what is known on the viral genomic variability in relation to clinical outcome.

1.5.1. CMV genomic variability

Several viral proteins have the capacity to modulate the host immune response (listed below). Therefore, the first attempt was to predict outcome by means of these immunomodulatory molecules.

- *UL144:* non-functional truncated TNF-α-like receptor gene;
- *US28:* functional β-chemokine receptor which binds and sequesters extracellular chemokines;
- *Envelope glycoproteins:* gB (UL55), gN (UL73), gH (UL75) have been associated with polymorphisms that may contribute escaping the immune response;
- *UL146, UL147:* α-chemokines, vCXCL-1 and vCXCL-2.

Some studies have related *UL144* (121, 122), *UL55 (gB)* (121, 123-125), *UL73 (gN)* (123, 126, 127), *UL146 (vCXCL-1)* and *UL147 (vCXCL-2)* (128, 129) to outcome, whereas others have not. For *US28* (121, 122) and *UL75 (gH)* (130, 131), there seems to be no clear association with cCMV outcome. Additionally, infection with multiple strains have been demonstrated in several populations: at the maternal-fetal interface (94), in congenitally infected newborns (132, 133), in immunocompetent and immunocompromised adults and young infants (134-138). Few studies have evaluated mixed infection in relation to clinical outcome, with some contradicting results (121, 130). However, in murine studies, and in immunocompromised patients, the mixed infection has been associated to enhanced pathogenicity (139-141). And unique genotypes are found in different compartments (133). How a mixed infection occurs in the fetus is largely unknown as it may occur as a single event with multiple strains, as multiple transmission moments or both mechanisms. To conclude, there are no strong evidence for either genomic variability nor for multiple strain infection in relation to short-term or long-term outcome.

1.5.2. Mothers

1.5.2.1. Predicting vertical transmission of CMV

Pregnant women who transmitted CMV to their fetus showed a lower and delayed lymphoproliferative response to CMV (78, 79). In these cohorts, the primary maternal infection occurred in different trimesters of gestation. The trimester of maternal primary infection is a determinant for vertical transmission, and the risk of transmission increases with the month of gestation (142, 143). In cases of infection in the first trimester, IgG avidity may be considered a prognostic marker for vertical transmission. A low avidity IgG (suggesting recent infection) is associated with 36% transmission, whereas intermediate avidity (timing of infection unclear) with 6% transmission (144). Contradicting results have been shown for maternal IgM, as it is used in different definitions of primary maternal infection. Indeed, when used as a marker for primary maternal infection the vertical transmission occurred only when IgM were present (145), while in another study of congenitally infected foetuses IgM could be detected only in 56% of mothers most likely because the group of mothers was characterized by both primary and secondary infection (though not stated clearly) (146). Overall, maternal seropositivity may be considered a marker for transmission. When a seronegative mother gets infected during pregnancy the vertical transmission rate is 30-35%, whereas in seropositive mothers this rate is lower (18-20). Concerning the virus, though very high viral load, evaluated by means of DNA quantification in AF, gave 100% of vertical transmission, very low or negative viral load, did not exclude it (145, 147). Different gB genotypes did not seem to correlate with transmission (148). Importantly, if the ultimate goal was to predict vertical transmission, all the mothers would need to be screened.

1.5.2.2. Predicting symptoms at birth

The gestational age at the onset of maternal primary infection is perhaps the most recognised factor related to cCMV outcome. If cCMV occurs in the first trimester the outcome is more severe, e.g. with neurological involvement, whereas if it occurs in the third trimester the symptoms, if any, are mild (149). Additionally, CMV DNA in AF was assessed in relation to maternal primary infection. Even though it seems that higher viral loads are related to a worse outcome at birth (145, 150-152), some studies have highlighted the importance of stratifying for onset of maternal primary infection. The CMV DNA in AF was significantly correlated to a worse outcome at birth if the maternal infection occurred relatively early (145, 149), whereas others have not found a correlation (153). Only few studies have evaluated the role of CMV DNA in AF of a group of mothers with both primary and secondary infection, and no correlation was found (147, 148). These findings may be due to different factors. First of all, primary maternal infection may result in higher AF viral load than secondary infection, as the maternal CMV-specific immune response is still developing and may not be able to control viral replication as efficiently as when a previous infection occurred. Second of all, a correlation between AF viral load and gestational age at the time of sampling has been shown (147, 148, 151), therefore when maternal primary infections occur early in gestation there might be an accumulation of viral DNA in AF that results in higher viral load (149).

1.5.2.3. Predicting LTI development

This has not been extensively explored, as after birth the immune system of the child may have a more important role in controlling CMV infection and disease. However, a trend towards higher risk of LTI development was shown for those infected neonates born from mothers with primary infection occurring in the first trimester of gestation (followed-up annually till 6 years of age) (149). This may be because earlier maternal primary infection infections are associated with symptoms at birth, and symptoms at birth are associated with LTI development. Whereas, CMV DNA in AF did not seem to correlate with LTI (149).

1.5.3. Fetus

1.5.3.1. Predicting symptoms at birth

Several markers, mainly quantified in cord blood, have been explored with the goal to predict clinical outcome, and these can be divided into different categories (virological, immunological, metabolic and imaging). In the majority of the cases, these markers have been quantified in the context of maternal primary infection. In case of secondary maternal infection this was specified.

Virological (markers related to viral components)

- *Antigenemia (detection of viral proteins in blood):* viral proteins are more indicative of a productive infection compared to DNA quantification, therefore they are associated with symptoms at birth (149, 152);
- *DNAemia (detection of viral DNA in blood):* the results are not conclusive in predicting symptoms at birth as some have found an association while others have not (149, 152, 154);
- *Viremia (detection of virus in blood):* the results are not conclusive in predicting symptoms at birth as some have found an association while others have not (149, 152).

Immunological (marker related to the host immune system)

- *Platelet counts:* lower platelet count has been consistently associated with symptomatic disease at birth (152, 154), and when combined with fetal ultrasound seemed to predict better (154);
- *IgM:* shown to be related to symptomatic disease at birth (149, 152);
- *White blood cells count:* higher levels were found in symptomatic (152);
- *% lymphocytes:* higher in the CMV+ foetuses and in those who were symptomatic at birth (152);
- β-*2 microglobulin:* higher in CMV+ foetuses and in those who were symptomatic at birth (152);
- *CD4+/CD8+:* even though the ratio is higher in CMV- no correlation was found in those CMV+ with symptoms at birth (152);
- *% CD3+HLADR+*: though higher in CMV+ there was no correlation with those who developed symptoms at birth (152).

Metabolic (markers related to the host metabolism)

- *Plasma aminotransferase:* aspartate aminotransferase (AST) increased in the symptomatic, whereas this was not observed for alanine aminotransferase (ALT) (152, 154, 155);
- *Gamma-glutamyl-transpeptidase (GGT):* no correlation was found in any of the studies, even though it was found higher in CMV+ foetuses (152, 154, 155).

Imaging

- *Ultrasound:* if fetal infection is not established, ultrasound may predict symptoms, whereas, if fetal infection is diagnosed, ultrasound imaging may be associated with a poor outcome (154, 156). In the absence of ultrasound abnormalities, a minority of neonates were symptomatic (154). The absence of abnormal ultrasound does not exclude the presence of abnormalities because some of them may appear later than when the ultrasound is performed;
- *MRI (Magnetic Resonance Imaging):* no correlation was found between MRI findings and symptoms in the first 11 months of life (157). This technique is more accurate and can show secondary lesions, even in the absence of ultrasounds abnormalities. Type of maternal infection not clear.

1.5.3.2. Predicting long-term impairment

The study of markers in fetus in relation to LTI development did not show any meaningful findings, though only few markers have been explored: CMV DNA, viremia, IgM, and ultrasound/MRI (149, 157). In particular, for imaging the main conclusion was that a normal ultrasound or MRI correlates with a normal development (157).

1.5.4. Neonate

1.5.4.1. Predicting symptoms at birth

Measuring markers at birth may have advantages over measuring them in the fetus because the material is more accessible and still reflects the situation during pregnancy, at least in the last phase of pregnancy. Therefore, a big overlap between markers evaluated in the fetus and in the neonates was observed, and a similar categorization is presented (virological, immunological and metabolic). In the majority of the cases, these markers were quantified in the context of maternal primary infection. In case of secondary maternal infection this is specified.

Virological

- *Antigenemia:* as in the fetus, higher levels are associated with symptomatic disease (158, 159);
- *DNAemia:* as in the fetus, contradicting results were shown (149, 158-161). In general, in all studies a big overlap between symptomatic and asymptomatic was observed, usually with

high viral load in both groups, and very low only in the asymptomatic. Therefore, what could be reliably concluded from these data is the lower risk of symptomatic disease in very low viral load (160). Interestingly, in a study of CMV DNA quantified in DBS, with an unknown type of maternal infection, higher viral loads correlated with a higher risk of SNHL (162). Importantly, the relationship between CMV DNA and symptoms is not linear, and this has been demonstrated not only in DBS (161-163), suggesting that the damage is evident after a certain accumulation of viruses;

- *CMV DNA in cerebrospinal fluid (CSF):* only found in symptomatic neonates (164);
- *CMV DNA in urine:* no correlation was found even though the viral load is usually higher in urine compared to blood ($10^{\wedge 5}$ vs $10^{\wedge 3}$), and persist longer (164).
- *Viremia:* associated with symptoms at birth (159);
- *Virus clearance (urine or blood):* the role of viral clearance in the clinical outcome remains not completely understood. Indeed, no difference in clearance from blood or urine was found between symptomatic and asymptomatic (161, 165). And there are some evidence of a shorter duration of excretion of CMV in urine in those neonates that had SNHL and progressive SNHL (165).

Immunological

- *IgM:* the results are discordant as some have not found a correlation (149, 159), whereas some have (166, 167);
- *β2-microglobulin and proteins:* found increased in symptomatic that developed LTI (168) (type of maternal infection not clear).

Metabolic

- *Platelet count + ALT:* with maternal infection not clear, if platelet count <100000/mm^3 + high ALT >80 UI/ml association with symptoms at birth (hearing loss) (169).

1.5.4.2. Predicting LTI development

In the majority of the cases, these markers were quantified in the context of maternal primary infection. In case of secondary maternal infection this was specified.

Virological

- *Antigenemia:* no correlation was found with respect to long-term outcome (149);
- *DNAemia:* the results are contradicting as some studies have not found a relation between load at birth in the neonatal blood and long-term outcome (149), whereas others have (170). A correlation between high loads in asymptomatic developing LTI compared to asymptomatic not developing LTI was observed (161).

Immunological

- *IgM:* no correlation was found between IgM in neonatal blood and LTI development (149);
- β*2-microglobulin and proteins:* higher in the CSF of symptomatic that developed LTI (168).

Clinical

- *Severe symptoms at birth (central nervous system (CNS) involvement):* are associated with higher risk of SNHL and cognitive deficits in a follow-up of ~4 years (maternal infection was not taken into consideration) (171). Clinical predictors are only available for symptomatic children;
- *Symptoms at birth*: studies have shown that even in case of not so severe symptoms at birth, symptomatic neonates have higher risk of developing LTI (17).

Imaging

- *CT (Computed Tomography):* abnormalities in symptomatic infected neonates are predictive for a worse long-term outcome (172), and in association with the presence of microcephaly at birth is predictive of cognitive LTI (173). In the latter, where all individuals were symptomatic, the absence of microcephaly in presence of CT abnormalities was associated with an intermediate outcome, whereas in absence of microcephaly and CT abnormalities with a good outcome.
- *MRI:* even though the spectrum of MRI abnormalities is extremely broad, there are certain abnormalities associated with a poor long-term outcome (age at MRI ~2 years and follow-up ~5 years) (174).

To conclude the section on prognostic markers for cCMV clinical outcome, there is no clear evidence of a biomarker that could potentially be used to define subgroups of patients that would benefit from certain clinical interventions. Several factors may have contributed to these discrepancies. First of all, the clinical definitions used for symptomatic neonates, or LTI development, are extremely diverse across studies. Different immunopathologies may underlie different outcomes. Second of all, in the majority of the cases a maternal primary infection was considered, therefore what happens in mothers with secondary infection is largely unknown. Third, it is almost impossible to determine the gestational age at vertical transmission.

1.6. CLINICAL STRATEGIES

Finally, even if we had "The Biomarker" allowing the identification of such subgroups, what, how, and to whom we offer certain clinical interventions that would solve/improve short- and long-term disabilities remain an open question. Such complex problem needs stratification according to the goals of the clinical intervention and to the target population of such an intervention. As a *primary prevention*, we would like to prevent maternal infection as cCMV would not occur at all, and this could be potentially achieved with a vaccine. As a *secondary prevention*, we would

like to prevent vertical transmission for the same aforementioned reason. However, there is no currently effective vaccine registered (175, 176), and there is no effective treatment that reduces vertical transmission, either antiviral or hyperimmunoglobuline (177, 178). As a *tertiary prevention*, we would like to prevent, or at least positively affect, the short- and/or long-term impairments. Provided that all infected neonates have been identified, e.g. through maternal and/or neonatal screening. Some studies have shown a certain degree of beneficial effect of a prenatal treatment with hyperimmunoglobuline or valacyclovir, however further studies are necessary (100, 179-182). Some other studies have focused on postnatal treatment of symptomatic neonates with (val) ganciclovir, and have shown an improvement of the hearing, language/receptive components (183-185). However, it still needs to be evaluated whether such treatment is beneficial for mildly affected or asymptomatic neonates who can still develop permanent impairments.

Importantly, several observations would need to be taken into consideration for the use of a future vaccine in the cCMV settings. Additionally to the mothers, infants and adolescents could be potential targets as well, depending on our goals. If given to infants it would prevent females to get infected and transmit the virus to the future foetuses. If given to toddlers we would reduce the transmission to mothers, though this would not be complete as the transmission from adults to adults may still occur. If given to adolescent girls we would prevent transmission to the fetus, similarly to when we give it to women, but with a different vaccine duration needed. The vaccine may as well be used in seropositive setting in order to boost pre-existing immunity.

As the way to a licensed vaccine seems to be long, the tertiary prevention sounds like a good compromise. However, in order this system to work a maternal/neonatal screening is necessary. The neonatal screening can be selective or universal. In the first case, CMV screening is reserved to those neonates that failed the hearing screening, or for those who have a specific clinical picture at birth. Therefore, all asymptomatic would be missed, as well as some symptomatic whose clinical signs are rather general and difficult to attribute to cCMV. In the second case, CMV screening is meant for all newborns, and is defined as "The systematic application of a test to identify asymptomatic individuals at risk of a specific disorder in order to prompt further investigation or preventative action. Benefits must outweigh harms." cCMV meets many criteria of the universal screening. However, the universal screening would identify those asymptomatic for whom no clear beneficial intervention plan is available. This would increase parental stress, alter the familiar dynamics (186), force treatments whose benefits are not established yet, and induce unnecessary medical exams (187). This problem would be considerably resized if predictors for clinical outcome were available.

Concluding, understanding the pathogenesis of cCMV would allow to figure out why certain children develop LTI and others do not. In turn, this would provide the necessary biomarkers to predict outcome and stratify patients according to the risk. On one hand we could define who benefits most from certain clinical interventions; and on the other hand we could define correlates of protection to be used in future vaccine trials.

1.7. OUTLINE ON THE THESIS

The aim of this thesis was to determine potential prognostic markers for short- and long-term clinical outcome, and to determine potential correlates of protection. The first would allow the identification of subgroups of children that would benefit from certain clinical interventions; whereas the latter could be useful for future vaccine development. These goals were achieved by using samples from a large retrospective nationwide cohort of children, with (n=125) and without (n=263) cCMV, born in The Netherlands in 2008 and their mothers.

In **Chapter 2** the immune system of the neonates, with and without cCMV, is assessed by means of quantification of several immunological markers extracted from dried blood spots (DBS) and correlated to the presence of cCMV, short- and long-term clinical outcome, and CMV viral load in DBS. These immunological markers refer to the number of γδ T cells, αβ T cells and B cells, with particular attention to B cell replication.

In **Chapter 3** the metabolism of the neonates, with and without cCMV, is assessed in relation to the presence of cCMV, short- and long-term clinical outcome, and CMV viral load. This is achieved by the analysis of the metabolic markers normally extracted from DBS after birth for the screening of the rare genetic metabolic disorders. The metabolic markers included are essential amino acids, hormones, carnitines and enzymes.

In **Chapter 4** the gene expression profile of the neonates, with and without cCMV, is assessed in relation to long-term clinical outcome, and CMV viral load. The RNA is extracted from DBS and sequenced by using the Illumina platform. Data analysis is performed with particular attention to the neonatal immune system.

In **Chapter 5** data on maternal and child HLA that are expressed at the placenta are presented. HLA-C, HLA-E, and HLA-G are determined from 96 mother–child pairs with cCMV in relation to short- and long-term clinical outcome, and CMV viral load. The mothers are additionally typed for killer cell immunoglobulin-like receptors (KIRs) to get more insights into NK cells response. The typing is performed on DNA extracted from buccal swabs from children and their mothers.

In **Chapter 6** data on maternal and child HLA that are not expressed at the placenta are presented. HLA-A, HLA-B, HLA-DR and HLA-DQ are determined from 96 mother-child pairs with cCMV in relation to a control group of 5604 Dutch blood donors and in relation to CMV viral load. The typing is performed on DNA extracted from buccal swabs from children and their mothers.

In **Chapter 7** a brief summary of the findings of each chapter is presented first, with particular attention to the mechanisms of cCMV pathogenesis and its clinical implications. Second, the main findings are integrated in one final model, with special emphasis on the future directions.

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CHAPTER 2

T AND B CELL MARKERS IN DRIED BLOOD SPOTS OF NEONATES WITH CONGENITAL CYTOMEGALOVIRUS INFECTION: B CELL NUMBERS AT BIRTH ARE ASSOCIATED WITH LONG-TERM OUTCOME

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ABSTRACT

Congenital cytomegalovirus infection (cCMV) is the most common congenital infection which can cause long-term impairment (LTI). The pathogenesis of LTI is not completely understood. Fetal immunity may play a role in controlling the infection and preventing LTI, though immune activation may also contribute to fetal immunopathology. In this study we analyzed various molecular markers of T and B cell numbers in neonatal Dried Blood Spots (DBS) of 99 children with cCMV and 54 children without cCMV: δRec-ψJα signal joints on T cell receptor excision circles, intronRSS-Kde signal joints on Igκ-deleting recombination excision circles, genomic intronRSS-Kde coding joint, genomic Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements. Of this cohort clinical symptoms at birth and LTI at 6 y of age were recorded. Neonates with cCMV had less TRECs in their blood than non-infected controls. Furthermore, cCMV infection was associated with increased numbers of γδ T cells and B cells, and these numbers were positively correlated with CMV viral load in the DBS. Infected children with a better long-term outcome had higher numbers of B cells at birth than those who developed LTI; no differences in B cell replication were observed. The potential protective role of B cells in controlling cCMV-related disease and the clinical value of this marker as a predictor of long-term outcome merit further evaluation.

2.1. INTRODUCTION

Human cytomegalovirus (CMV) is the most common cause of congenital infections worldwide with an overall birth prevalence in industrialized countries between 0.6-0.7% (1, 2). Among congenitally infected children, 12.7% are estimated to be symptomatic at birth with the most common symptoms being petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly (1, 2). An estimated 40-58% of these symptomatic children will develop permanent sequelae, such as hearing loss, mental retardation, and developmental delay (1). Importantly, out of the 87.3% of neonates that are asymptomatic at birth, approximately 13.5% will develop permanent sequelae as well (1). Defining markers that may help to predict whether a neonate will develop long-term impairment (LTI) will have profound impact on postnatal policy.

The pathogenesis of fetal damage during congenital infection is still poorly understood due to the complex interplay between viral, maternal, placental and fetal factors. It has been shown that infections occurring predominantly in the first half of pregnancy are associated with sequelae (3, 4). The developing fetal immune system may play a role in controlling the infection later in pregnancy, thereby preventing the development of long-term sequelae (5). There is evidence of fetal and neonatal immune system activation in the context of congenital CMV infection (cCMV). Previous studies have shown expansions of fetal γδ T cells (6), $CD8^+$ T cells (7, 8) and $CD4^+$ T cells (7, 9) in cCMV. Upon congenital infection similar types of effector $CD4^+$ and $CD8^+$ T cells as in adults are generated (10-12) but they appear to be functionally impaired (7), the $CD4^+$ T cell response being more impaired than the $CD8^+$ T cells (8, 13). Despite the fetal capacity to generate IgM against CMV (14), the fetal B cell response has not been extensively studied in cCMV. Previous studies on cellular and humoral immunity in cCMV have not related their findings to either symptoms at birth or LTI.

In recent years, molecular markers for T and B cells have been used to address several clinical questions. These markers specifically involve the circular excision products produced upon the most common TCR and Ig gene rearrangements during T and B progenitor cell generation in thymus and bone marrow, respectively. δRec-ΨJα signal joints on TCR excision circles (TRECs) are used in newborn screening on dried blood spots (DBS) for primary immunodeficiency such as SCID (15, 16) because they can be readily detected by quantitative PCR (17-19). Furthermore, studies have been carried out to implement screening for agammaglobulinemia (20), using quantitative PCR on DBS for Igκ-deleting recombination excision circles (KRECs). In addition, measurement of TRECs has been used in HIV patients to monitor the cellular recovery after initiation of highly active antiretroviral therapy (21), and in stem cell transplantation patients with primary immunodeficiency, TRECs and KRECs were used to monitor the thymic T cell and bone marrow B cell neogenesis (22, 23). Finally, the replication history of isolated B cell subsets has been used to improve the characterization of immunological disease with aberrant B lymphoid maturation (24-26).

For this study, neonatal DBS from a large cohort of children with cCMV and from non-infected controls were analyzed for molecular markers of T and B cells and the results were related to clinical data from birth until 6 y of age. Using quantitative real time PCR on DNA isolated from DBS, TCR and Ig gene rearrangements were detected. δRec-ΨJα signal joints on TRECs were detected as a measure of αβ T cell thymogenesis and coding joints of Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements

in the genome as a measure of circulating γδ T cells. Furthermore, intronRSS-Kde coding joints (cj intronRSS-Kde) in the genome and intronRSS-Kde signal joints on KRECs were quantified as a measure of circulating B cells and newly-derived bone marrow B cells, respectively. KRECs were additionally used to determine the B cell replication history (27).

In the present study, the quantification of TRECs and KRECs, as well as molecular markers for γδ T cells, was applied to provide new insights into the immune regulation of cCMV and to identify early markers to predict LTI at 6 y of age, such as neurodevelopmental impairment.

2.2. MATERIALS AND METHODS

2.2.1. Study population and clinical data

A previously described, nationwide, retrospective cohort was used in this study. This cohort was derived from a total group of 31,484 children, born in 2008 in the Netherlands, that was retrospectively tested for cCMV infection by PCR for CMV DNA in neonatal DBS at 5 y of age (28). After approval by the Medical Ethics Committee of the Leiden University Medical Center, the parents of 125 congenitally CMV infected children and of 263 non-infected children were asked to participate. Parents of 99 congenitally infected children gave informed consent for the use of DBS and the use of clinical data for this study. In addition, 54 controls without cCMV were randomly sampled from a control group matched for gender-, month-of-birth and region. Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or purpura, hypotonia, abnormal laboratory findings (elevated liver transaminases, hyperbilirubinemia, neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domain (hearing, visual, neurological, motor, cognitive and speech-language). Additionally, the severity of the LTI was assessed by accumulating the number of domains affected and indicated as the presence of LTI in two or more domains. The same definitions were used for children with and without cCMV. Finally, in this cohort maternal seroimmunity to CMV before birth was unknown, therefore it was assumed that cCMV infection could have resulted from either maternal primary or secondary infection.

2.2.2. DNA extraction from DBS and quantitative PCR for CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment, a second confirmatory PCR was performed at the Leiden University Medical Center (28). For this purpose, DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (29). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR as described previously (30, 31) on a CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate and the CMV viral load expressed in IU/ml.

2.2.3. Quantification of TCR and Ig gene rearrangements

To study the numbers of T and B cells in neonatal DBS, the most frequently formed TCR and Ig gene rearrangements were quantified by TaqMan-based quantitative PCR. The δRec-ΨJα rearrangement occurs in 70-80% of TCRD alleles in mature αβ T lymphocytes, resulting in TRECs in nearly all newly formed αβ T cells (32). Vδ1-Jδ1 and Vδ2-Jδ1 rearrangements are collectively present in nearly all mature γδ T cells in neonates, but absent in αβ T cells (6, 33). The intronRSS-Kde rearrangement occurs in ~30% of Igκ$^+$ and in almost all Igλ$^+$ mature B lymphocytes (27).

These rearrangements as well as the β-globin housekeeping gene were quantified in triplicate from each sample using two multiplex real-time PCR assays (Table 1). Each multiplex assay consisted of 5 µl of DNA extract and 20 µl reaction mixture containing 3.5 mM of $MgCl_2$, 0.04 mg/ml of BSA and 12.5 µl HotStar Master mix (QIAGEN, Hilden, Germany). Both mixtures were optimized by primer limitation, and for probe and $MgCl_2$ concentration with primers and probes specific for TREC, KREC, cj intronRSS-Kde, Vδ1-Jδ1, Vδ2-Jδ1 and β-globin to ensure equal amplification efficiencies. The Phocine Herpes Virus (PhHV) amplification was used to check for inhibition of the PCR and β-globin amplification was used to control for the number of nucleated cells. The following PCR mixes were used: the first mix contained 300 nM primers with 200 nM probe for cj intronRSS-Kde, Vδ1-Jδ1 and 500 nM primers with 200nM probe for β-globin. The second mix contained 300 nM KREC, Vδ2-δ1 and PhHV primers, 900 nM TREC primers, 200 nM KREC, TREC and Vδ2-δ1 probes, 50 nM PhHV probe.

Quantification was performed using dilution series of DNA from the following cell lines: DB01+T, a modification of the previously published U698 DB01 cell line (27) that contains one TREC, one KREC and one cj intronRSS-Kde rearrangement copy per genome; Peer (34), a T lymphoid cell line, containing one Vδ1-Jδ1 rearrangement copy per genome and T-ALL T032 (34), a T-acute lymphoblastic leukemia cell line, containing one Vδ1-Jδ1 rearrangement construct per genome. For TRECs two positive controls (cord blood with two different levels of TRECs spotted on filter paper) and a negative control (leukocyte-reduced adult blood produced by filtration and spotted on filter paper) were included in each run. These materials were kindly provided by the Centers for Disease Control and Prevention, Atlanta. The real-time PCR was performed on a CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands) in a 96-well plate using a thermocycling profile as follows: 15 min 95°C followed by 45 cycles of 95°C (30 s), 55°C (30 s) and 72°C (30 s). Data were analyzed with CFX Manager 3.1 software.

The percentage of cells that contained the rearrangement in relation to the total amount of nucleated cells in blood was calculated using the ΔΔCt method with the formula as previously described (35): $2^{((CT_{\beta\text{-globin}} - CT_{target})_{sample} - (CT_{\beta\text{-globin}} - CT_{target})_{cell\ line})}*100\%$. The housekeeping gene β-globin was quantified in both the sample and cell line used as standard.

The average number of B cell divisions, B cell replication history, was calculated with the formula as previously described (35): $(CT_{sj} - CT_{cj})_{sample} - (CT_{sj} - CT_{cj})_{cell\ line}$.

The copies/µl of whole blood were calculated based on the theoretical recovery of 1 µg DNA from approximately 150,000 cells (19, 36) and the assumption that one full spot contains 50 µl of whole blood.

Table 1. Primers and probes used for the two internally-controlled multiplex rea- time PCRs.

	Targets	Forward primer (5'- 3')	Reverse primer (5'- 3')	Probe (5'- 3')
Mix I	cj[a] int-Kde (144 bp) [Ref (35)]	CCCGATTAATGCTGCCGTAG	CCTAGGGAGCAGGGAGGCTT	FAM[b]-AGCTGCATTTTTGCCATATCCACTATTTGGAGT-BHQ[c]-1
	Vδ1-Jδ1 (200-300 bp) [Ref (33)]	ATGCAAAAAAGTGGTCGCTATT	TTAGATGGAGGATGCCTTAACCTTA	TXR[d]-CCCGTGTGACTGTGGAACCAAGTAAGTAACTC-BHQ-2
	β-globin (97 bp)	AAGTGCTCGGTGCCTTTAGTG	ACGTGCAGCTTGTCACAGTG	YAK[e]-TGGCCTGGCTCACCTGGACAACCT-BHQ-1
Mix II	sjf KREC int-Kde (148 bp) [Ref (35)]	TCAGCGCCCATTACGTTTCT	GTGAGGGACACGCAGCC	YAK-CCAGCTCTTACCCTAGAGTTTCTGCACGG-BHQ-1
	Vδ2-Jδ1 (around 200 bp) [Ref (33)]	ATACCGAGAAAAGGACATCTATG	TTAGATGGAGGATGCCTTAACCTTA	TXR-CCCGTGTGACTGTGGAACCAAGTAAGTAACTC-BHQ-2
	sj TREC ΨJα -δRec (131 bp) [Ref (35)]	CCATGCTGACACCTCTGGTT	TCGTGAGAACGGTGAATGAAG	FAM-CACGGTGATGCATAGGCACCTGC-BHQ-1
	PhHV[g] (89 bp)	GGGCGAATCACAGATTGAATC	GCGGTTCCAAACGTACCAA	CY5[h]-TTTTTATGTGTCCGCCACCATCTGGATC-BHQ-1

[a] coding joint; [b] 6-carboxyfluorescein; [c] Black Hole Quencher; [d] Texas Red; [e] Yakima Yellow; [f] signal joint; [g] phocine herpesvirus type 1; [h] Cyanine.

2.2.4. Sensitivity and efficiency of the quantitative PCR assays

Ten-fold serial dilutions of DNA from the various control cell lines carrying the target rearrangements were used to determine the assay sensitivity. The analytical sensitivity, expressed as the lower limit of detection, was assessed by testing the dilutions in triplicate. Quantification of β-globin levels was possible with 0.05 ng total DNA with fewer than 36 PCR cycles in DB01+T, Peer and T-ALL 032 cell lines. Quantification of TREC and KREC levels was possible with 0.05 ng total DNA with fewer than 37 PCR cycles. Quantification of cj intronRSS-Kde, Vδ1-Jδ1 and Vδ2-Jδ1 levels was possible with 0.05 ng total DNA with < 36 PCR cycles.

The similar efficiency between each target and the β-globin is required to use the ΔCt method for correct quantification of normalized targets levels (37-39). The amplification efficiency, determined from the slope of the log-linear portion of the calibration curve, was assessed by testing the dilutions in triplicate. The efficiencies of the assays were very similar: 0.99 ± 0.02 for cj intronRSS-Kde, 0.98 ± 0.01 for KREC, 0.96 ± 0.02 for TREC and 0.99 ± 0.01 for β-globin in DB01+T cell line; 1.04 ± 0.01 for Vδ1-Jδ1 and 1.03 ± 0.03 for β-globin in Peer cell line; 1.06 ± 0.03 for Vδ2-Jδ1 and 0.98 ± 0.01 for β-globin in T-ALL 032 cell line; all $R^2 \geq 0.98$.

2.2.5. Statistics

The differences in the levels of immunological markers between the different categories - CMV status, viral load, symptoms at birth and LTI - were assessed by using a linear mixed model with random effects in order to account for the repeated measurements on the same patient. A Pearson's correlation analysis between viral loads, log(IU/ml), and the different immunological markers was carried out. P-values <0.05 were considered statistically significant. Due to the exploratory nature of this study the correction for multiple comparison was not applied for multiple statistical testing. Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL)**.**

2.3. RESULTS

2.3.1. Study population and clinical data

DBS of 99 children with cCMV were tested for using two multiplex real-time PCR assays (Table 1), as were 54 controls. The study population and the presence of symptoms at birth and LTI are shown in Table 2. In the control group, seven (12.9%) children showed symptoms at birth and five (9%) had LTI. In the children with cCMV, 16 (16%) children were symptomatic at birth and 22 (22%) had LTI.

2.3.2. TCR and Ig gene rearrangements in DBS of children with cCMV versus controls

First, the effect of cCMV was assessed by quantifying TCR and Ig gene rearrangements on DNA from DBS using real time PCR and comparing children with cCMV (cCMV+) and children without cCMV (cCMV-). Supplemental Table 1 shows the estimated means for the markers in our cohort. The cCMV+ group had a trend towards significant decrease percentage of cells that contained

Table 2. Long-term impairments in the group with cCMV and controls.

	Congenital CMV infection			No congenital CMV infection		
Long term impairment	**n = 99**	**Asympt.[a] n = 83**	**Sympt.[b] n = 16**	**n = 54**	**Asympt.[a] n = 47**	**Sympt.[b] n = 7**
Hearing impairment[c]	1	1	0	0	0	0
Visual impairment[d]	2	2	0	0	0	0
Neurological impairment[e]	4	2	2	4	4	0
Motor impairment[f]	10	7	3	1	1	0
Cognitive impairment[g]	4	2	2	2	2	0
Speech/language problem[h]	15	9	6	3	3	0
One or more impairment[i]	22	14	8	5	5	0
More than one impairment[j]	7	5	2	3	3	0

[a] Asymptomatic (Asympt.) at birth; [b] Symptomatic (Sympt.) at birth; [c] Sensorineural hearing loss; [d] Optic nerve atrophy, cortical visual impairment, congenital cataract; [e] Cerebral palsy, epilepsy, microcephaly, ADHD, autism; [f] Motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder; [g] Cognitive impairment based on test or diagnosis; [h] Language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder; [i] Any long-term impairment, in one or more domains; [j] Impairment in two or more domains.

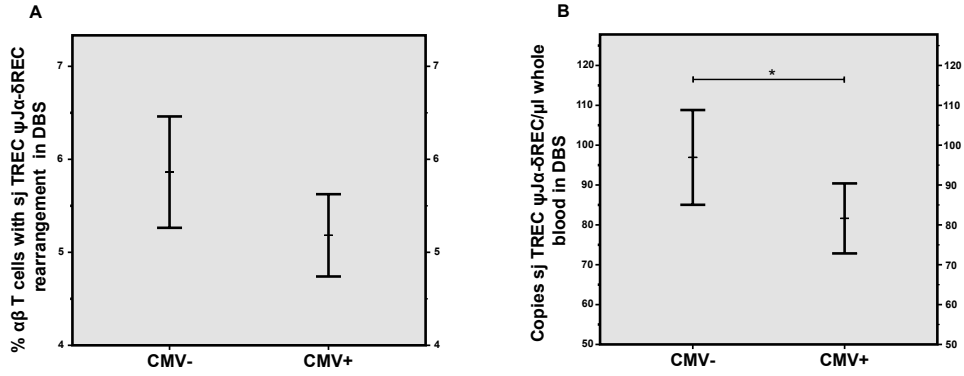

Figure 1. TRECs in DBS of CMV infected children and uninfected controls. (**A**) Frequency of TREC rearrangements in the group with cCMV (CMV+) and controls (CMV-). p = 0.073. (**B**) Absolute TREC numbers per µl of whole blood. The estimated means from each group of patients ± 2 SE are shown. *p = 0.043.

the TREC rearrangement normalized for the presence of β-globin (p = 0.073) (Fig. 1A). In accordance with this finding, the number of TRECs per µl of whole blood was lower than the control group (p = 0.043) (Fig. 1B).

It has been shown that prematurity, defined as birth before 37 wk gestational age, is related to a lower amount of TRECs (15, 40) and that intra-uterine growth retardation, or dysmaturity, is associated with a small thymus (41). To exclude their influence on the differences in TRECs' levels, an additional sensitivity analysis was performed by first excluding the group of premature newborns, 10 (10%) with cCMV and 4 (7.4 %) in the control group, and then by excluding the group of dysmature

newborns, 2 (2%) with cCMV and 2 (3.7%) in the control group. No differences were found in the estimates and significance compared to the whole cohort (data not shown). Therefore, our data suggest that the reduction in the amount of TRECs is not confounded by prematurity or by being small for gestational age.

The percentage of γδ T cells that contained the Vδ1-Jδ1 rearrangement was significantly higher in the cCMV+ group (p = 0.019). To exclude the possibility that this higher percentage of Vδ1-Jδ1 rearrangements was the results of fewer αβ T cells, we determined the absolute number of Vδ1-Jδ1 per microliter of whole blood. Indeed, significantly more Vδ1-Jδ1 copies per µl of whole blood were present in the infected group (p = 0.038). No statistically significant difference between the groups was observed for the percentage of cells with the Vδ2-Jδ1 rearrangement nor for the Vδ2-Jδ1 copies per µl of whole blood.

In the cCMV+ group, the percentage of cells that contained the cj intronRSS-Kde rearrangement was higher than in the cCMV- group (p = 0.055). As the cj intronRSS-Kde copies per µl of whole blood were slightly higher in the cCMV+ group, but not statistically significant, the small increase in percentage might be due to a decrease in αβ T cells. The KREC copies and the percentage of cells that carried a KREC were not different between the cCMV+ and cCMV- groups (p = 0.297, p = 0.139 respectively) even though the same trend of higher numbers in the cCMV+ group was observed. Consequently, the B cell replication history was not significantly different between cCMV+ and controls.

2.3.3. TCR and Ig gene rearrangements in DBS of children with cCMV in relation to CMV viral load

To study the relation between T and B cell numbers with CMV viral load, the cCMV+ group was divided into three by taking two cut-points at the first and third quartile of the viral load in DBS, resulting in a low (n=24), medium (n=50) and high (n=25) viral loads groups. The mean viral loads for each group were 254, 2907 and 27121 IU/ml, and the estimated means for the molecular T and B cell markers according to these groups are shown in Table 3.

The high viral load group did not show statistically significant differences compared with the low and medium viral load groups for the TRECs copies per µl whole blood or the percentage of cells that contained the TREC rearrangement.

DBS from neonates with high CMV viral loads showed a significantly higher percentage of cells containing Vδ1-Jδ1 rearrangements than the low viral load group (p = 0.022), and slightly higher than the medium viral load group (p = 0.124) (Fig. 2A). In addition, the Vδ1- Jδ1 copies per µl of whole blood were significantly higher in this group than in the low and medium viral load groups (p = 0.033, p = 0.041, respectively) (Fig. 2B). Furthermore, a Pearson's *r* data analysis suggested a positive correlation between Vδ1- Jδ1 percentage as well as Vδ1- Jδ1 copies per microliter, and viral loads (p < 0.001, r = 0.25, and r = 0.23, respectively). No statistically significant differences between the groups were observed for the percentage of cells that carried Vδ2-Jδ1 rearrangements nor in the copies Vδ2- Jδ1 copies per microliter of whole blood.

Table 3. Estimated means and SEs of all immunological markers in viral load.

	Viral load cCMV infection		
Estimated means (SE)	**Low[a] (n = 24)**	**Medium[b] (n = 50)**	**High[c] (n = 25)**
T cell markers			
TREC (%)	5.2 (0.5)	5.3 (0.3)	5.0 (0.4)
TREC copies/µl	79 (9)	81 (6)	83 (8)
Vδ1-Jδ1 (%)	0.42 (0.08)	0.53 (0.05)	0.68 (0.08)
Vδ1-Jδ1 copies/µl	10 (2)	12 (1)	17 (2)
Vδ2- Jδ1 (%)	0.6 (0.08)	0.71 (0.06)	0.71 (0.08)
Vδ2-Jδ1 copies/µl	19 (2)	20 (2)	22 (2)
β-glob copies/µl	2307 (141)	2304 (98)	2439 (138)
B cell markers			
KREC (%)	2.6 (0.3)	2.7 (0.2)	4.0 (0.3)
KREC copies/µl	34 (5)	38 (3)	65 (5)
cj int-Kde (%)	2.06 (0.31)	2.37 (0.21)	3.06 (0.3)
cj int-Kde copies/µl	27 (4)	32 (3)	48 (4)
B rep. history	-0.39 (0.09)	-0.23 (0.06)	-0.4 (0.09)

[a] Viral loads below first quartile; [b] Viral loads between first and third quartile; [c] Viral loads above third quartile.

The percentage of cells that contained KRECs (Fig. 2C) and the KREC copies per microliter (Fig. 2D) were significantly higher in the high viral load group than in the low and medium viral load groups ($p = 0.002$, $p = 0.001$, and $p<0.001$, respectively). A Pearson's *r* data analysis suggested a positive correlation between percentage of KREC, as well as KREC copies per microliter, and viral load ($p < 0.001$, $r = 0.33$, and $r=0.41$, respectively). Additionally, in the high viral load group, the percentage of cells that contained the cj intronRSS-Kde rearrangement was significantly higher than the low viral load group ($p = 0.024$), and trend significantly higher than the medium viral load group ($p = 0.068$) (Fig. 2E). These differences were even more significant for the cj intronRSS-Kde copies per µl of whole blood ($p = 0.001$, $p = 0.004$, respectively) (Fig. 2F). A Pearson's *r* data analysis suggested a positive correlation between percentage cj intronRSS-Kde, as well as cj intronRSS-Kde copies per microliter, and viral loads ($p < 0.01$, $r = 0.26$, and $r=0.37$, respectively). No differences in the B cell replication history were observed between the viral load groups.

2.3.4. TCR and Ig gene rearrangements in DBS of children in relation to symptoms at birth

Next, TCR and Ig gene rearrangements were studied in relation to symptoms at birth to evaluate whether they reflected differences in symptoms (Supplemental Table 1). The comparison of T and B cell markers between asymptomatic cCMV+ and symptomatic cCMV+ individuals did not show statistical differences for any of the markers. Additionally, when comparing the asymptomatic cCMV+ with asymptomatic cCMV- groups a similar trend was observed in the same markers as

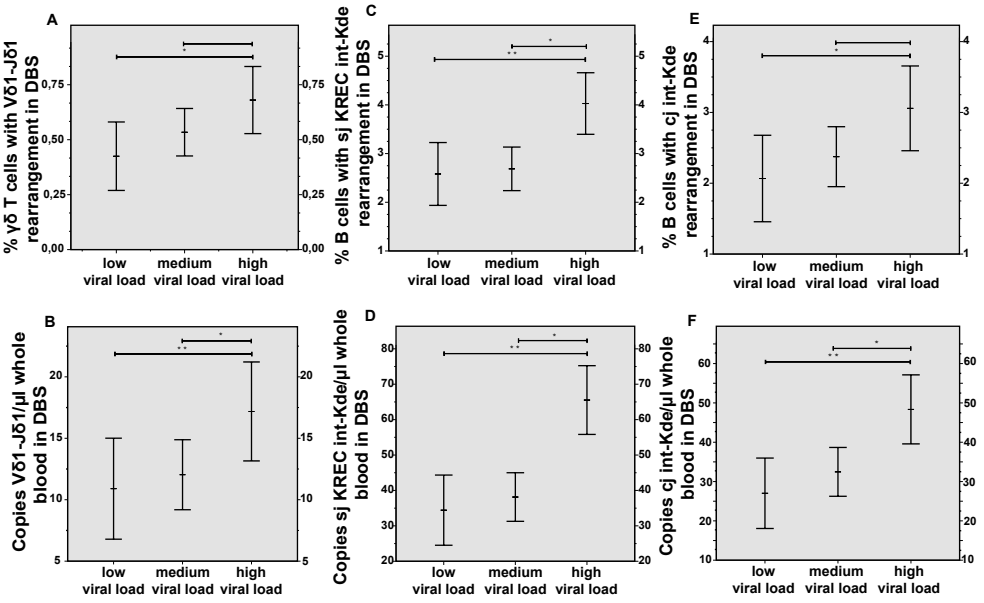

Figure 2. Vδ1-Jδ1, sj KREC int-Kde, and cj intronRSS-Kde in DBS of CMV infected children with different viral loads. (**A**) Frequency of Vδ1-Jδ1 rearrangements in the infected group (CMV+) with low, medium and high viral load on DBS. *p = 0.022. (**B**) Absolute Vδ1-Jδ1 numbers per microliter of whole blood in the infected group (CMV+) with low, medium and high viral load on DBS. *p = 0.041, **p = 0.033. (**C**) Frequency of sj KREC int-Kde rearrangements in the infected group (CMV+) with low, median and high viral load on DBS. *p = 0.001, **p = 0.002. (**D**) Absolute sj KREC int-Kde numbers per µl of whole blood in the infected group (CMV+) with low, median and high viral load on DBS, both p < 0.001. (**E**) Frequency of cj intronRSS-Kde rearrangements in the infected group (CMV+) with low, median and high viral load on DBS. *p = 0.024. (**F**) Absolute cj intronRSS-Kde numbers per µl of whole blood. *p = 0.004, **p = 0.001. The estimated means from each group of patients ± 2 SE are shown.

shown in the overall comparison between cCMV+ and cCMV- children (data not shown). Finally, no significant differences in viral loads were found between symptomatic and asymptomatic subjects.

2.3.5. TCR and Ig gene rearrangements in DBS of children in relation to long-term impairment

Next, the TCR and Ig gene rearrangements were correlated with LTI (Supplemental Table 1). First, we evaluated the immunological markers in relation to the development of any disorder in one or more of the following domains of impairment: hearing, visual, neurological, motor, cognitive and speech-language. When comparing the group of children with cCMV infection that develop any LTI to those who do not, a significantly lower percentage of cells that contain the KREC rearrangement was observed (p = 0.008) (Fig. 3A). Also the KRECs copies per µl of whole blood were significantly lower in the patients with LTI (p = 0.005) (Fig. 3B). A similar trend, although not statistically significant, was observed for the percentage of cells that contained the cj intronRSS-Kde

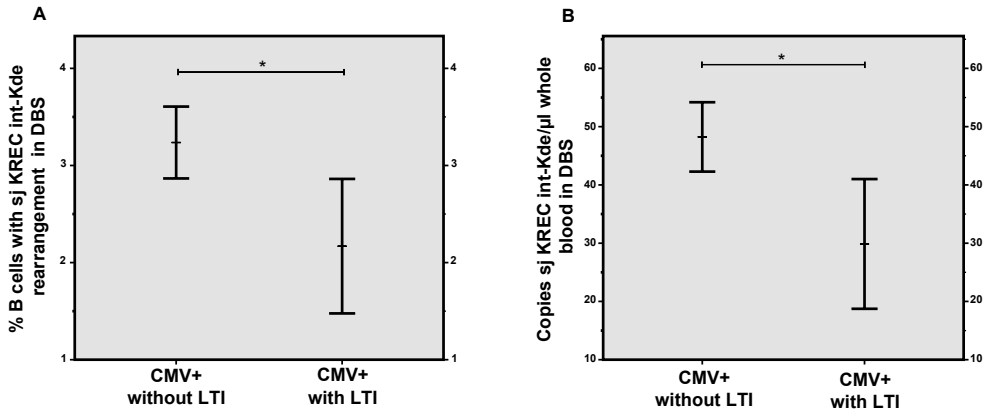

Figure 3. B cells and long-term impairments in cCMV infected children. (**A**) Frequency of sj int-Kde KREC rearrangement on DBS in the cCMV+ group without and with any long term impairment (LTI). *p = 0.008. (**B**) Absolute sj KREC int-Kde numbers per microliter of whole blood on DBS in the cCMV+ group without and with any LTI. *p = 0.005.

rearrangement as well as cj intronRSS-Kde copies per µl of whole blood (p = 0.137, p=0.073). Second, TCR and Ig gene rearrangements were assessed in relation to the development of more extensive impairments, defined as LTI in two or more domains. Similar trends were observed when children with more extensive impairments were compared to children without any LTI. Lower percentages and numbers of KRECs (p = 0.04, p = 0.02, respectively) and slightly lower percentages and numbers of cells containing cj intronRSS-Kde rearrangements were detected in cCMV+ children with more extensive LTI than in cCMV+ children without any LTI. No differences in B cell replication history and TCR rearrangements were observed in relation to LTI.

Next, we evaluated if the same markers that were significant in the overall cCMV+ and cCMV- comparison were still present in absence of any LTI. Indeed, a similar trend was observed in the same markers, TREC and Vδ1-Jδ1 (data not shown). In addition, the cCMV+ group had a significant increase in the percentage of cells that contains the KREC rearrangement (p = 0.021) and KREC copies (p = 0.044) as well as percentage of cells that contains the cj intronRSS-Kde rearrangement (p = 0.017) and slightly higher cj intronRSS-Kde copies per microliter of whole blood (p = 0.075).

Finally, no significant differences in viral loads were found between congenitally infected children with and without any LTI.

2.4. DISCUSSION

The analysis of molecular markers for T and B cells from DBS in this large cohort of children shows that cCMV resulted in reduced thymic production of αβ T cells, increased numbers of γδ T cells and a trend towards increased numbers of B cells. Children with cCMV and LTI did show lower number of B cells. The observed trend of B cells number increase in the infected group was further emphasized when excluding the patients with LTI, who had a lower number of B cells and might have diluted the effect.

The reduced number of TRECs in the DBS from children with cCMV suggests that intrauterine infection leads to reduced thymic production of T cells. Indeed, the sensitivity analysis indicated that the reduction in the amount of TRECs in our cohort is not confounded by prematurity or by being small for gestational age. CMV infection is known to induce a shift from naïve towards more differentiated αβ T cells with a reduction in the pool of naïve T cells, as has been shown in immunosuppressed individuals and in elderly (42, 43) as well as in pregnant CMV IgG-seropositive women (44). However, these all concern adults in whom the reduction of the pool of naïve T cells might result from a process that takes place over a longer period of time than the gestational period and where the longevity of naïve T cells might also play a role in masking differences in thymic output, with the reduced output being visible only after several years (45). Therefore, this process is unlikely to be the cause of the reduced amount of TREC in our cohort. Interestingly, in vitro CMV has been shown to be capable of infecting thymic epithelial cells that play a central role in T cell development and maturation, both during gestation and early stage of life (46, 47). Moreover, in newborn infants with cCMV, hypoplastic thymuses have been described and both in guinea pig and mouse models pathologic changes of the thymus have been shown (48-50). Although the TREC numbers were only moderately lowered, the effect of cCMV on thymopoiesis certainly deserves further study.

In our study we did not find an increase in Vδ2 T cells in the cCMV infected group, however, we looked at Vδ2-Jδ1 so a role of γδ T cells with other Vδ-Jδ rearrangements, as reported previously, cannot be excluded (6). Our observation of increased percentages of Vδ1 T cells in children with cCMV is in accordance with a previous study that showed a γδ T cell response upon cCMV after primary maternal CMV infection. These fetal γδ T cells, detected as early as 21 wk of gestation, were shown to be activated, to undergo cell division and to become differentiated with highly restricted repertoires (6). Moreover, γδ clones derived from cCMV-infected newborns showed antiviral activity when incubated with CMV-infected cells (6) suggesting a role in controlling viral replication (51). These unconventional cells, react rapidly upon activation (52) and develop earlier than αβ T cells during immune ontogeny. Therefore, they might have an important role in early life (51) and, possibly, in a context where the αβ T-cell response is impaired, they might be more efficient in controlling the early phases of cCMV. In the mouse model, when adaptive mechanisms are impaired or absent, γδ T cells can provide effective control over CMV infection (53, 54). To further support this, γδ T cell expansion in solid organ transplanted patients in response to CMV was associated with the resolution of infection and less symptomatic CMV disease, whereas late γδ T cell expansion correlated with a more intense and durable CMV infection (55). In our cohort, this was not associated with fewer symptoms at birth or LTI. However, the symptoms that define CMV disease in solid organ-transplanted patients are different to the clinical signs in the cCMV setting and the ability of γδ T cells to control long-term CMV disease, has not been elucidated (52).

CMV viral load in DBS was not correlated to symptoms at birth or to LTI in our cohort. Some previous studies have demonstrated a relation between CMV viral load with clinical outcome (56, 57), whereas others have not (58-60). The predictive role of CMV viral load in blood for congenital CMV disease may differ depending on the timing of infection and whether there was primary maternal infection or recurrent infection and therefore it still needs to be clarified. In our cohort

it is impossible to establish the trimester of infection or if a maternal primary infection occurred. Interestingly, none of our tested molecular B and T cell markers were associated with symptoms at birth in cCMV infected children. cCMV infected children with LTI did show significantly lower absolute and relative numbers of KRECs as well as slightly decreased, though not statistically significant, absolute and relative numbers of cj intronRSS-Kde, compared with cCMV infected children without LTI. However, there were no differences in B cell replication. The same trend was observed when considering more severe LTI. These findings suggest that cCMV infection does not induce a notable intrauterine B cell proliferation and possibly no considerable Ab production, but rather hint to an increase in B cell production. Unfortunately, little information is available on fetal B cell immunity in relation to cCMV. IgM positive B cells have been shown to emerge in the peripheral circulation as early as 12 wk of gestation (61) and CMV infected fetuses can produce IgM (62-64), but the antiviral activity and the role in CMV disease control have not yet been evaluated (5). Whether the different numbers of B cells are associated with postnatal differences in the capacity to generate long-lived plasma cells, memory B cells or support effector functions of immune cells remains to be elucidated. The potential protective role of Ab can be illustrated by the fact that primary CMV infection in pregnancy is associated with a vertical transmission rate of 30-40% and that this risk is at least 10-fold-lower in seropositive pregnant women (2). Although it is uncertain whether Abs are capable of influencing an ongoing CMV infection, a possible better initial B cell response might be beneficial in controlling the progressive tissue damage responsible for LTI development, possibly due to a sustained viral replication and spread. A positive correlation between B cells number and viral load was observed. In a group of pregnant women with primary infection, an expansion of a large pool of activated memory B cells enriched for CMV specificity and higher in viremic women was shown, further supporting a causal relationship between high viral loads and cells activation (65). On the other hand, the difference in B cell numbers that we observed at birth between infected children with and without LTI may also be related to a different timing of infection, with earlier infections leading to a more extensive cCMV and inflammation influencing the early B cell lymphopoiesis in the fetal liver.

To our knowledge, this is the first study on molecular markers for T and B cells in neonatal DBS of cCMV infected children in relation to long-term outcome. A reliable marker for long-term outcome could provide the means to introduce the long debated (66) newborn screening program for CMV in DBS by defining subgroups that would benefit from clinical, audiological follow-up and possibly antiviral treatment. Whether KREC, that was related to LTI, has enough discriminative power needs to be assessed in other CMV cohorts. Finally, this study on molecular markers generates new hypotheses on the effects of CMV infection on fetal, and possibly child, immunity and on the potential protective role of B cells in cCMV infection.

2.5. ACKNOWLEDGEMENTS

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2.6. DISCLOSURES

The authors have no financial conflicts of interest.

2.7. FUNDING

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Table S1. Estimated means and standard errors of all immunological markers in the different categories

	Congenital CMV infection						No congenital CMV infection					
Estimated means (SE)	**Overall n = 99**	**Asympt.[1] n = 83**	**Sympt.[2] n = 16**	**No LTI[3] n = 77**	**LTI (≥ 1)[4] n = 22**	**LTI (≥ 2)[5] n = 7**	**Overall n = 54**	**Asympt. n = 47**	**Sympt. n = 7**	**No LTI n = 49**	**LTI (≥ 1) n = 5**	**LTI (≥ 2) n = 3**
T cell markers												
TREC (%)	5.2 (0.2)	5.2 (0.2)	5.0 (0.6)	5.2 (0.3)	5.1 (0.5)	4.9 (0.8)	5.9 (0.3)	6.0 (0.3)	4.7 (0.8)	5.8 (0.3)	6.9 (0.9)	5.6 (1.2)
TREC copies/µl	81 (4)	82 (5)	78 (11)	82 (5)	82 (9)	69 (16)	96 (6)	99 (6)	84 (17)	96 (6)	103 (20)	80 (26)
Vδ1-Jδ1 (%)	0.54 (0.03)	0.56 (0.04)	0.45 (0.1)	0.56 (0.05)	0.51 (0.08)	0.56 (0.15)	0.40 (0.05)	0.44 (0.03)	0.15 (0.09)	0.39 (0.04)	0.56 (0.12)	0.35 (0.15)
Vδ1-Jδ1 copies/µl	13 (1)	14 (1)	10 (3)	14 (1)	11 (2)	10 (4)	9 (1)	11 (1)	4 (2)	9 (1)	13 (3)	8 (4)
Vδ2- Jδ1 (%)	0.68 (0.04)	0.68 (0.05)	0.66 (0.10)	0.65 (0.05)	0.77 (0.9)	0.81 (0.16)	0.77 (0.06)	0.79 (0.06)	0.66 (0.16)	0.77 (0.06)	0.79 (0.19)	0.61 (0.24)
Vδ2-Jδ1 copies/µl	20 (1)	21 (2)	19 (4)	20 (2)	24 (3)	23 (5)	23 (2)	24 (2)	18 (6)	23 (2)	21 (7)	17 (9)
[6]β-glob copies/µl	2339 (70)	2344 (76)	2314 (173)	2339 (79)	2339 (147)	2014 (259)	2450 (94)	2421 (103)	2639 (267)	2461 (102)	2340 (318)	2275 (410)
B cell markers												
KREC (%)	3.0 (0.2)	2.9 (0.2)	3.1 (0.4)	3.24 (0.19)	2.17 (0.35)	1.8 (0.6)	2.6 (0.2)	2.6 (0.2)	2.8 (0.5)	2.55 (0.19)	3.1 (0.6)	2.8 (0.8)
KREC copies/µl	44 (2)	44 (3)	46 (7)	48 (3)	30 (6)	22 (10)	39 (3)	39 (3)	44 (9)	38 (3)	50 (10)	36 (13)
cj int-Kde (%)	2.5 (0.1)	2.5 (0.17)	2.5 (0.40)	2.6 (0.17)	2.0 (0.33)	1.9 (0.6)	2.0 (0.2)	2.01 (0.16)	2.04 (0.42)	1.96 (0.16)	2.56 (0.49)	2.28 (0.63)
cj int-Kde copies/µl	35 (2)	35 (3)	34 (6)	37 (3)	27 (5)	24 (9)	30 (3)	31 (3)	30 (8)	30 (3)	39 (9)	31 (12)
B rep. history	-0.31 (0.04)	-0.31(0.05)	-0.34 (0.12)	-0.36 (0.05)	-0.16 (0.09)	-0.03 (0.17)	-0.38 (0.06)	-0.37(0.05)	-0.43 (0.14)	-0.39 (0.05)	-0.33 (0.17)	-0.30 (0.22)

[1] Asymptomatic at birth; [2] Symptomatic at birth; [3] No long-term impairment in any of the domain: hearing, visual, neurological, motor, cognitive and speech-language; [4] Any long-term impairment, in one or more domain; [5] Impairment in two or more domains; [6] biallelic β-globin copies.

CHAPTER 3

NEONATAL SCREENING PARAMETERS IN INFANTS WITH CONGENITAL CYTOMEGALOVIRUS INFECTION

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ABSTRACT

Congenital Cytomegalovirus infection (cCMV) is the most common cause of congenital infections worldwide that can cause long-term impairment (LTI). The metabolic alterations due to cCMV are largely unknown. This study aims to assess the metabolites included in the neonatal screening in relation to cCMV and cCMV outcome, allowing the identification of prognostic markers for clinical outcome. Essential amino acids, hormones, carnitines and enzymes from dried blood spots (DBS) were analyzed of 102 children with cCMV and 179 children without cCMV, and they were related to symptoms at birth and LTI at 6 years of age. In this cohort, the neonatal screening parameters did not change in relation to cCMV, nor to symptoms at birth or LTI. However, metabolic changes were observed in children born preterm, with lower concentrations of essential amino acids in premature infants with cCMV compared to premature controls. Finally, a higher concentration of palmytoilcarnitine (C16) in the group with higher viral load was observed. Though these data demonstrate limitations in the use of neonatal screening data as predictors for long-term cCMV outcome, the metabolism of preterm neonates with cCMV merits further evaluation.

3.1. INTRODUCTION

Cytomegalovirus (CMV) infection is a common infection with a seroprevalence of almost 50% in the general Dutch population (1). CMV is the most common cause of congenital infections worldwide with an overall birth prevalence of 0.6-0.7% in industrialized countries (2, 3). A significant part of children with congenital CMV infection (cCMV) will have long-term permanent neurological sequelae. Among congenitally CMV-infected children, 12.7% is estimated to be symptomatic at birth with the most common symptoms being petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly (2, 3). An estimated 40-58% of these symptomatic children will develop permanent sequelae, such as hearing loss, mental retardation, and developmental delay (3). Approximately 13.5% of the asymptomatic children will develop permanent sequelae as well (3).

cCMV outcome is the result of a complex interplay between viral, maternal, fetal, and child factors. *In vitro*, CMV has been shown to influence different cellular metabolic pathways. The fatty acid biosynthetic pathway of infected cells is highly upregulated in order to sustain the viral envelope production (4-6). In infected cells, the increased glucose uptake and glycolytic activity provide the necessary carbon atoms used for fatty acid biosynthesis (4, 6, 7). The cellular energy requirement is then insured by the increase of glutaminolysis in order to allow the tricarboxylic acid cycle (TCA) to function (8). *In vivo*, few studies have evaluated the metabolic changes occurring in children with cCMV. A recent metabolomics analysis on amniotic fluid (AF) showed that primary CMV infection during pregnancy, irrespective of fetal infection, resulted in the activation of glutamine-glutamate and pyrimidine metabolic pathways and, when comparing asymptomatic CMV-infected newborns to symptomatic CMV-infected newborns, a possible shift in fatty acid biosynthesis was observed (9). Moreover, in a group of congenitally infected children a metabolic fingerprint was identified in urine samples compared to uninfected controls. An increase of ketone bodies (3-hydroxybutyrate and 3-aminoisobutyrate) was observed in the CMV-infected group in an attempt to compensate a general reduced level of ATP (10).

The aim of this study was to assess the metabolites included in the neonatal screening, which is performed in Dried Blood Spots (DBS), in relation to cCMV and cCMV outcome. Importantly, several considerations should be taken into account with respect to the neonatal screening, specifically designed to diagnose rare genetic metabolic disorders. First of all, excluding the metabolic disorders, changes in these metabolites have only been reported in critically ill children. A decrease in thyroid hormones in septic neonates with poor outcome was observed and premature critically ill neonates showed different amino acids profiles, usually with higher concentrations, compared to the healthy controls (11, 12). The majority of newborns with cCMV do not have symptoms at birth or only have mild disease, and the clinical signs of symptoms and LTI included in this cohort are diverse. Therefore, if any changes in metabolites are found, these will most likely be subtle. Second, several factors have been described influencing the analytes measured on DBS, such as fetal blood volume, hematocrit, gestational age, birth weight, maternal factors and storage conditions (13-19). However, despite these potential limitations, this exploratory study was undertaken to study biomarkers in neonatal DBS of cCMV-infected children. This could allow the identification of prognostic markers

for long term outcome of cCMV with a profound impact on parental counselling, postnatal interventions and the potential introduction of neonatal screening for cCMV. For this purpose, the neonatal screening data of a large nation-wide cohort of children with and without cCMV was evaluated in relation to long-term impairment (LTI) at the age of six years.

3.2. MATERIALS AND METHODS

3.2.1. Study population and clinical data

A previously described, nationwide, retrospective cohort was used for this study. A group of 31,484 children born in 2008 in the Netherlands was retrospectively tested for cCMV by PCR for CMV DNA in neonatal DBS at 5 years of age (20). cCMV was diagnosed in 156 children and informed consent for retrieval of medical data was given by parents of 133 children with cCMV and 274 matched controls. After approval by the Medical Ethics Committee of the Leiden University Medical Center, the parents of 102 congenitally CMV-infected children and 197 children without cCMV gave informed consent to retrieve the neonatal screening data of their child. The controls without cCMV are from a gender-, month-of-birth and region-matched control group. Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or pupura, hypotonia, abnormal laboratory findings (elevated liver transaminases, hyperbilirubinemia, neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domain (hearing, visual, neurological, motor, cognitive and speech-language). Because in this cohort maternal seroimmunity to CMV before birth was unknown, it was assumed that cCMV infection could have resulted from either maternal primary or recurrent infection.

3.2.2. DNA extraction from DBS and qPCR of CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment (RIVM), a second confirmatory PCR was performed at the Leiden University Medical Center (LUMC) (20). For this purpose, DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (21). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR as described previously (22, 23) on a CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate, and the CMV viral load expressed in IU/ml.

3.2.3. Neonatal screening data

The genetic metabolic disorders included in the Dutch neonatal screening program in 2008 are listed in Table 1. The screening is carried out on DBS and involves five regional accredited screening laboratories, among which the National Institute for Public Health and the Environment (RIVM). The DBS are collected between 72 and 168 hours after birth and the markers are quantified mainly

Table 1 Genetic disorders included in the Dutch neonatal screening program in 2008.

Disorder	Marker	Quantification method	Incidence[1]
Amino acid disorders			
Glutaricaciduria type 1 (GA I)	C5DC	MS/MS	1: 335455
Isovaleric academia (IVA)	C2, C5	MS/MS	1: 351429
Maple syrup urine disease (MSUD)	Leucine, Valine	MS/MS	1:567692
Homocystinuria (HCU)	Met	MS/MS	1:167727
3-methylcrotonyl-CoA- carboxylase Deficiency (3-MCC)	C5OH	MS/MS	1: 194211
HMG-CoA lyase deficiency	C5OH	MS/MS	1:100000 [34][2]
multiple CoA carboxylase deficiency (MCD)	C5OH	MS/MS	1:200000 [35]
Phenylketonuria (PKU)	Phenylalanine, Tyrosine	MS/MS	1: 11865
Fatty acid oxidation disorders			
Medium chain acylCoA dehydrogenase Deficiency (MCAD)	C8, C10	MS/MS	1: 23730
Long-chain hydroxyacyl-CoA dehydrogenase Deficiency (LCHAD)	C16OH	MS/MS	1:410000
Very long chain acylCoA dehydrogenase Deficiency (VLCAD)	C14:1, C16	MS/MS	1: 144706
Carnitine transporter deficiency (CTD)	C0	MS/MS	1:40000 [36]
Endocrine disorders			
Congenital hypothyroidism (CH)	T4, TSH, TBG	Immunochemistry	1:3000 – 1:4000 [37]
Congenital adrenal hyperplasia (CAH)	17-OHP	AutoDELFIA	1:10000 – 1:20000 [38]
Other			
Galactosemia (GAL)	GALT, TGAL	Enzymatic method	1: 49530
Biotinidase deficiency (BTD)	BIOT	Enzymatic method	1: 49865

[1] Unless otherwise specified the incidence is retrieved from the Dutch Diagnosis Registration Metabolic Diseases (DDRMD) database [39]; [2] for HMG-CoA lyase deficiency the prevalence is reported.

using Tandem Mass Spectrometry (MS/MS) or Immunochemistry as reported in Table 1. The data were provided by the National Institute of Public Health and the Environment (RIVM), Department of Prevention Programs. The cut off values of the parameters used for referral of the child are described in the national guideline for neonatal screening (24). An analyte below or above the cut-off levels would strongly indicate the presence of a rare genetic metabolic disorder, though a confirmatory test would be needed on a second DBS. Thus, the whole cohort of children included in the study was screened for analytes above or below the cut-off levels. Furthermore, among the factors described to influence the metabolites measured on DBS, gestational age and birthweight are the most important and well-characterized (13). These factors were taken into account in the analysis, and the markers were first assessed in premature versus term infants, and then in dysmature versus non-dysmature infants, within the whole cohort. Prematurity was defined as birth before 37 weeks of gestation while dysmaturity as weight at birth less than -2 SD for gestational age. Then, the analysis

of the metabolites in relation to cCMV and cCMV outcome was subsequently stratified for these factors in order to evaluate if they were of significant influence.

3.2.4. Statistics

The differences in the levels of the metabolic markers between the different categories - cCMV status, CMV viral load, symptoms at birth, and LTI - were assessed by using an independent-samples t-test. A Chi-square test was used to assess the differences in the proportion of prematures and dysmatures in relation to cCMV and LTI. Several analytes including C5OH, C5DC, C5 and C16OH showed results below the limit of reliable quantification and were therefore excluded from the analysis. The carnitines included in the present study (C0, C2, C8, C10, C14:1 and C16) identify fatty acid disorders associated to different pathways which are short, medium, long and very long chain fatty acids metabolism. Therefore, in order to gain more insights into these pathways total carnitine (TC), acylcarnitines molar ratio (AFR), short chain index (SCI), medium chain index (MCI) and long chain index (LCI) were additionally calculated as previously described (25), with slight modifications according to the carnitine available in this study. TC was calculated by addition of all available acylcarnitines (AC) and free carnitine (FC); AFR was calculated as the ratio between AC/FC; SCI as C2 divided by TC level; MCI as the sum of C8 and C10 divided by TC level; LCI as the C14 and C16 divided by TC level. A p value <0.05 was considered statistically significant. Due to the exploratory nature of this study, no multiple comparison correction was applied. Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL, USA).

3.3. RESULTS

3.3.1. Study population and clinical data

The clinical data of the study population are shown in Table 2. Neonatal screening data were retrieved from 102 children with cCMV and 179 non-infected controls. In the control group, 12.4% of children (n = 22) had symptoms at birth and 8.4% (n = 15) showed any LTI. In the children with cCMV, 17.6% (n = 18) had symptoms at birth and 24.5% (n = 25) showed LTI. In this cohort, 10.8% of children with cCMV (n = 11) and 5.1% of children without cCMV (n = 9) were born preterm, while 2% of children with cCMV (n = 2) and 5.6% of children without cCMV (n = 10) were born dysmature, these differences were not significant. However, a significantly higher percentage of prematures was observed in the cCMV-infected group that developed LTI compared to cCMV-infected group that did not (24% and 6.5% respectively, p = 0.024), whereas this was not observed in the control group (data not shown). Additionally, within the cCMV-infected group, all children born premature with birthweight <2500 g developed LTI, and only one premature child with birthweight ≥ 2500 g did so, whereas none of the non-infected premature neonates developed LTI.

3.3.2. Neonatal screening data in relation to congenital CMV infection

First, the metabolic markers were assessed in relation to cCMV. For this purpose, CMV-positive children (n = 102) were compared with CMV-negative children (n = 179), and no significant differences were found in any of the markers included in this study (data not shown).

Table 2. Clinical data in the group with cCMV and controls.

	Congenital CMV infection			No congenital CMV infection		
	Overall n (%) N = 102	Asympt.[1] n (%) N = 84	Sympt.[2] n (%) N = 18	Overall n (%) N = 179[3]	Asympt. n (%) N = 156	Sympt. n (%) N = 22
Gender						
Male	60 (58.8)	48(57.1)	12 (66.7)	96 (53.6)	83 (53.2)	12 (54.5)
Female	42 (41.2)	36 (42.9)	6 (33.3)	83 (46.4)	73 (46.8)	10 (45.5)
Gestational age (weeks)[4]						
<32	1 (1.0)	0 (0.0)	1 (5.6)	2 (1.1)	0 (0.0)	2 (9.1)
32 - < 37	10 (9.8)	0 (0.0)	10 (55.6)	7 (3.9)	0 (0.0)	7 (31.8)
≥ 37	91 (89.2)	84 (100)	7 (38.9)	169 (94.4)	156 (100)	13 (59.1)
Birthweight (g)[5]						
<1500	1 (1.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)
1500-2499	5 (5.0)	0 (0.0)	5 (27.8)	10 (5.6)	0 (0.0)	10 (45.4)
≥2500	95 (94.1)	83 (100)	12 (66.7)	168 (94.4)	156 (100)	12 (54.5)
Premature	11 (10.8)	-	11 (61.1)	9 (5.1)	-	9 (40.9)
Dysmature	2 (2.0)	-	2 (11.1)	10 (5.6)	-	10 (45.5)
Long term impairment						
Hearing impairment[6]	3 (2.9)	2 (2.4)	1 (5.6)	0 (0.0)	0 (0.0)	0 (0.0)
Visual impairment[7]	3 (2.9)	3 (3.6)	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)
Neurological impairment[8]	5 (4.9)	3 (3.6)	2 (11.1)	8 (4.5)	8 (5.1)	0 (0.0)
Motor impairment[9]	11 (10.8)	8 (9.5)	3 (16.7)	2 (1.1)	2 (1.3)	0 (0.0)
Cognitive impairment[10]	4 (3.9)	2 (2.4)	2 (11.1)	2 (1.1)	2 (1.3)	0 (0.0)
Speech/language impairment[11]	17 (16.7)	10 (11.9)	7 (38.9)	9 (5.0)	8 (5.1)	1 (4.5)
One or more impairment[12]	25 (24.5)	15 (17.9)	10 (55.6)	15 (8.4)	14 (9.0)	1 (4.5)

[1] Asymptomatic at birth; [2] Symptomatic at birth; [3] For one CMV-negative child data on symptoms at birth were not available; [4] For one CMV-negative child gestational age was not available; [5] For one CMV-positive and one CMV-negative child birthweight was not available; [6] sensorineural hearing loss; [7] optic nerve atrophy, cortical visual impairment, congenital cataract; [8] cerebral palsy, epilepsy, microcephaly, ADHD, autism; [9] motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder; [10] cognitive impairment based on test or diagnosis; [11] language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder; [12] Any long-term impairment, in one or more domains.

Furthermore, as previously mentioned, gestational age and birthweight are important and well known factors influencing the metabolites measured on DBS. Because of the significant percentage of premature and dysmature infants in the group with symptoms at birth, we aimed to evaluate if these variables affected the metabolic markers in this cohort. For this purpose, we compared neonates born premature (n = 20) with non-premature (n = 260), and dysmature neonates (n = 12) with non-dysmature neonates (n = 267) using the total cohort, both children with and without cCMV. A significantly decreased concentration of T4 and biotinidase activity (BIOT) was observed in the premature group compared to non-premature, as well as a significantly increased 17OHP concentration (Table 3) ($p < 0.001$). No differences in concentration were found between dysmature neonates and non-dysmature neonates (data not shown). Given the diversity of clinical signs in the symptomatic children, we next wondered whether prematurity was the main factor driving

Table 3. Metabolic markers in association with prematurity.

	All children[1]			Preterm children[2]			Preterm children with cCMV[3]		
Mean (SE)	Term (n = 260)	Preterm (n = 20)	p-value	cCMV- (n = 9)	cCMV+ (n = 11)	p-value	No LTI (n = 5)	LTI (n = 6)	p-value
Essential amino acids[4]									
Methionine	17.8 (0.3)	19.5 (1.3)	0.150	21.1 (2.2)	18.2 (1.5)	0.269	19.2 (1.2)	17.3 (2.6)	0.564
Leucine	155.4 (2.6)	140.7 (10.0)	0.132	167.7 (15.0)	118.6 (9.5)	0.010	134.6 (16.3)	105.3 (8.8)	0.132
Valine	127.2 (2.2)	114.7 (8.7)	0.128	129.0 (14.7)	102.9 (9.3)	0.137	118.8 (7.4)	89.7 (14.2)	0.121
Phenylalanine	64.7 (1.1)	73.0 (4.0)	0.038	81.7 (6.7)	65.9 (3.7)	0.044	65.8 (2.0)	66.0 (6.9)	0.980
Hormones[5]									
T4[6]	83.7 (1.1)	68.5 (5.0)	<0.001	62.2 (6.7)	73.6 (7.0)	0.267	81.6 (5.4)	66.8 (11.9)	0.321
17OHP[7]	0.81 (0.02)	1.2 (0.1)	<0.001	1.3 (0.2)	1.2 (0.1)	0.399	1.13 (0.06)	1.18 (0.14)	0.769
Carnitines[8]									
C0[9]	17.5 (0.5)	19.3 (1.6)	0.276	21.3 (3.1)	17.7 (1.4)	0.273	16.7 (2.4)	18.6 (1.7)	0.538
C2[10]	19.9 (0.5)	23.3 (2.1)	0.094	25.1 (3.6)	21.7 (2.4)	0.428	19.4 (2.2)	23.7 (4.0)	0.400
C8[11]	0.034 (0.001)	0.036 (0.004)	0.494	0.034 (0.005)	0.037 (0.006)	0.726	0.030 (0.004)	0.043 (0.010)	0.295
C10[12]	0.050 (0.001)	0.056 (0.006)	0.367	0.05 (0.01)	0.06 (0.01)	0.190	0.05 (0.01)	0.07 (0.01)	0.332
C14:1[13]	0.061 (0.003)	0.07 (0.01)	0.362	0.07 (0.02)	0.07 (0.01)	0.823	0.08 (0.02)	0.06 (0.01)	0.344
C16[14]	2.72 (0.06)	2.29 (0.22)	0.053	2.3 (0.4)	2.3 (0.3)	0.880	2.5 (0.4)	2.1 (0.4)	0.461
Enzymes[15]									
BIOT[16]	99.3 (1.4)	85.2 (3.2)	<0.001	78.7 (3.9)	90.5 (4.4)	0.067	101.8 (5.3)	81.0 (3.7)	0.009
GALT[17]	100.5 (1.6)	94.1 (4.8)	0.277	86.7 (8.1)	100.1 (5.4)	0.170	92.4 (9.6)	106.5 (5.1)	0.207

[1] Metabolic markers in relation to prematurity; [2] Metabolic markers in relation to cCMV in premature neonates; [3] Metabolic markers in relation to LTI in cCMV-infected premature neonates; [4] The concentration of essential amino acids is given in µmol/l of blood; [5] The concentration of hormones is given in nmol/l of blood; [6] T4 = Thyroxine; [7] 17OHP = 17-Hydroxyprogesterone, values in log scale; [8] The concentration of carnitine is given in µmol/l of blood; [9] C0 = Free carnitine; [10] C2 = Acetylcarnitine; [11] C8 = Octanoylcarnitine; [12] C10 = Decanoylcarnitine; [13] C14:1 = Tetradecenoylcarnitine; [14] C16 = Palmitoylcarnitine; [15] The enzyme activity is given in % compared to the average of the daily run; [16] BIOT = biotinidase activity; [17] GALT = galactose-1-phosphate uridylyltransferase.

the aforementioned metabolic changes, or rather a combinations of clinical signs. Hence, an additional sensitivity analysis was performed by first comparing the group of symptomatic neonates (n = 40) with the asymptomatic neonates (n = 240) without excluding premature neonates. Then the group of premature was excluded, and symptomatic neonates (n = 20) were compared to asymptomatic neonates (n = 240). The results and significance changed suggesting that the observed differences were mainly driven by prematurity (data not shown).

Therefore, we next assessed the markers in relation to cCMV by stratifying for prematurity. Premature cCMV-negative children (n = 9) were compared to premature cCMV-positive children (n = 11), and term cCMV-negative (n = 169) children were compared to the term cCMV-positive (n = 91). The premature cCMV-positive showed lower levels of essential amino acids compared to premature controls, statistically significant for leucine and phenylalanine (p = 0.01 and p = 0.04 respectively) (Table 3 and Fig. 1). No differences were found when comparing term cCMV-positive with term cCMV-negative (Fig. 1).

Next, in order to assess the influence of CMV viral load on the metabolic markers, the cCMV-positive group was divided into two groups according to the median viral load in DBS, which was 3.1 log (IU/ml), namely low (n = 50) and high viral load (n = 50) group. The high viral load group had significantly higher concentration of palmitoylcarnitine (C16) (p = 0.002) (Table 4 and Fig. 2). This was not influenced by the presence of premature neonates (data not shown). As a

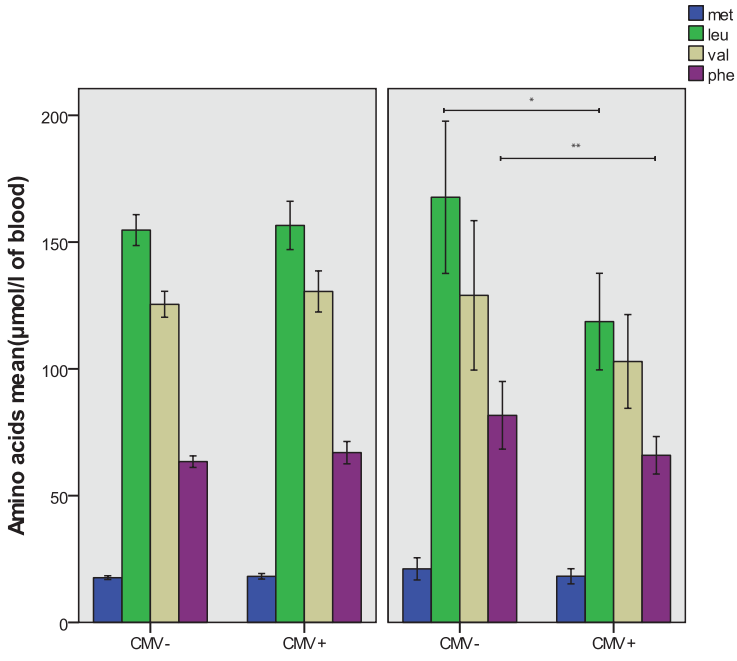

Figure 1. Essential amino acids in relation to cCMV in term and preterm infants. *Left panel:* essential amino acids in CMV-negative and CMV-positive term infants (n=169 and n=91, respectively). *Right panel:* essential amino acids in CMV-negative and CMV-positive pre-term infants (n=9 and n=11, respectively). The mean from each group of infants ± 2SE are shown. *p = 0.01 and **p = 0.04.

Table 4. Metabolic markers in relation to CMV viral load.

	Viral Load cCMV infection[1]		
Mean (SE)	**low load[2] (n=50)**	**high load[3] (n=50)**	**p-value**
Essential amino acids			
Methionine	18.4 (0.8)	18.2 (0.7)	0.846
Leucine	157.7 (6.9)	147.2 (6.1)	0.255
Valine	128.1 (5.2)	127.2 (5.9)	0.911
Phenylalanine	65.5 (2.5)	68.7 (3.2)	0.431
Hormone			
T4	79.9 (2.9)	87.5 (3.1)	0.075
17OHP	0.8 (0.1)	0.91 (0.04)	0.082
Carnitines			
C0	18.1 (1.3)	17.6 (1.0)	0.764
C2	19.0 (1.4)	22.5 (1.6)	0.113
C8	0.033 (0.002)	0.033 (0.003)	0.951
C10	0.049 (0.002)	0.054 (0.003)	0.208
C14:1	0.06 (0.01)	0.06 (0.01)	0.961
C16	2.4 (0.2)	3.1 (0.1)	0.002
Enzymes			
BIOT	99.3 (3.2)	99.3 (2.8)	0.993
GALT	99.0 (3.4)	102.8 (4.1)	0.471
Pathways			
TC[4]	39.81 (2.61)	43.42 (2.47)	0.318
AFR[5]	1.28 (0.06)	1.51 (0.06)	0.008
SCI[6]	0.48 (0.01)	0.51 (0.01)	0.029
MCI[7]	0.0023 (0.0001)	0.0021 (0.0001)	0.164
LCI[8]	0.066 (0.003)	0.076 (0.003)	0.008

[1] CMV viral load measured in DBS. For two infected neonates the DBS was not available, therefore CMV viral load could not be assessed. [2] CMV viral load below the median (3.1 log (IU/ml)); [3] CMV viral load above the median (3.1 log (IU/ml)); [4] Total carnitine (TC); [5] Acylcarnitines molar ratio (AFR); [6] Short chain index (SCI); [7] Medium chain index (MCI); [8] Long chain index (LCI).

result, the acylcarnitine molar ratio (AFR) and the long-chain index (LCI) were increased (both p = 0.008) (Table 4).

Finally, the genetic metabolic disorders, included in the neonatal screening, were assessed in relation to cCMV. Six children without cCMV showed an increased 17-Yhydroxyprogesteron (17OHP) concentration. However, when taking into account the gestational age, only two infants had dubious 17OHP results. Furthermore, one child with cCMV showed a low concentration of free carnitine (C0). The analysis of clinical data of these infants did not show any underlying disorders related to congenital adrenal hyperplasia nor to carnitine transporter deficiency. Therefore, none of the metabolites' concentrations are affected by any of the genetic conditions included in this neonatal screening.

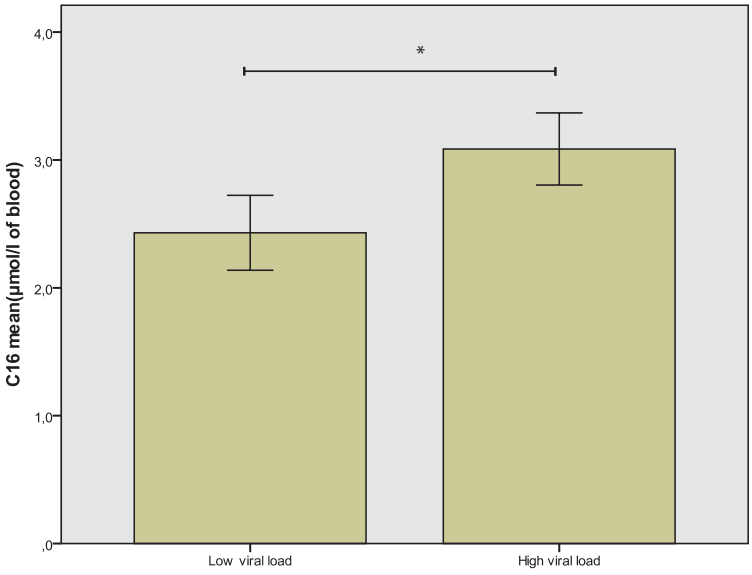

Figure 2. Palmytoilcarnitine (C16) in relation to CMV viral load. *Low viral load:* CMV-positive infants with viral load below the median measured in DBS (n=50). *High viral load:* CMV-positive infants with viral load above the median measured in DBS (n=50). The mean from each group of infants ± 2SE are shown. *p = 0.002.

3.3.3. Neonatal screening data in relation to long-term impairment

Next, neonatal screening data were analysed in the group of children with cCMV in relation to LTI development in one or more of the following domains of impairments: hearing, visual, neurologic, motor, cognitive, and speech-language. Given the previously shown effect of prematurity on different analytes, the analysis was stratified for this factor, and infected premature neonates who developed LTI (n = 6) were compared to infected premature neonates without LTI (n = 5). A lower BIOT was observed in the neonates who developed LTI compared to those who did not (p = 0.009) (Table 3), while this was not observed when comparing infected term neonates who developed LTI (n = 19) with infected term neonates without LTI (n = 72) (data not shown). Finally, in order to establish whether a relationship between the metabolites and LTI development may occur independently of cCMV, the same analysis was performed in the control group. The difference between non-infected premature neonates that developed LTI and those who did not, could not be assessed because none of the nine prematures developed LTI. Whereas no differences were observed when comparing non-infected term neonates who developed LTI (n = 15) with non-infected term neonates who did not (n = 154).

3.4. DISCUSSION

Few studies have evaluated the metabolic effect of cCMV, and this is the first exploratory study on biomarkers in neonatal DBS of cCMV infected children in relation to long-term outcome (9, 10). A reliable biomarker for cCMV long-term outcome that does not require additional neonatal tests

could provide the means to introduce the long debated newborn screening program for cCMV in DBS (26). Indeed, this would define subgroups of children that would benefit from clinical, audiological follow-up and possibly antiviral treatment.

cCMV outcome is the result of a multifactorial process that accounts for viral, maternal, fetal, and child factors. The host metabolism has already been shown to be altered following CMV infection, both *in vivo* and *in vitro* (4, 6-10), therefore it may play a role in cCMV outcome. This exploratory study shows that, overall, there were no differences in concentrations of metabolic and endocrine parameters included in the neonatal screening between cCMV-positive children and controls. Likewise, between cCMV-infected children who developed LTI and cCMV-infected children who did not, suggesting that these neonatal screening data cannot be used as predictors for long-term outcome in the general population. The effects we demonstrated were detected when prematurity was taken into account and, though the numbers of individuals may be a limiting factor, if confirmed in other cohorts, these findings give useful insights into metabolic changes in relation to cCMV in infected preterm neonates. The main metabolic changes concerned lower concentration of essential amino acids in premature cCMV-infected newborns (Fig. 1).

Importantly, gestational age and birthweight are the best characterized variables responsible for the variation of metabolites, mainly attributed to fetal stress and immature functions of liver and kidney. A lower concentration of T4, BIOT and an increased concentration of 17OHP has already been demonstrated in preterm infants (27-31). In this cohort, similar variations were found, indicating that these changes are attributable to prematurity rather than the infection.

Moreover, we aimed to evaluate whether higher CMV viral loads may alter the host metabolism more efficiently than lower viral loads, and found a significantly higher concentration of C16 in the former (Fig. 2). Though this may be a coincidental finding, the relationship between CMV infection and palmitic acids was described before. In a study of pregnant women with primary CMV infection, the palmitic acids was found decreased in AF of transmitters mothers as well as increased in AF of symptomatic neonates (9). Palmitate (16:0), which is the end product of fatty acid synthase pathway, is the precursor of longer chain fatty acids. The CMV envelope is enriched for longer chain fatty acids and its infectivity is reduced by the inhibition of fatty acid elongases (ELOVLs), an enzyme involved in their biosynthesis (32). Therefore, the increase of C16 in the high viral load group may simply reflect the increased viral burden.

Finally, because in this cohort maternal seroimmunity to CMV before birth and trimester of vertical transmission were unknown, the influence of these conditions could not be assessed. Therefore, significant metabolites differences, in relation to cCMV and cCMV outcome, cannot be entirely excluded if such conditions were taken into account. Nevertheless, this cohort study, retrieved from a large population screening, does reflect a real population of newborns with cCMV in all its diversity, ranging from no symptoms at birth and no LTI to symptoms at birth with severe LTI. Furthermore, in view of the described differences in sensitivity of the CMV PCR on DBS (33), it is important note that with the high sensitivity of our PCR (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (20). Therefore, the influence of the sensitivity of the CMV PCR on DBS on our conclusions can be considered negligible.

In conclusion, although these findings demonstrate limitations in the use of routine neonatal screening data as predictors for long-term cCMV outcome, a possible influence of cCMV in the amino acids metabolism of preterm neonates, and of higher viral loads in the metabolism of longer chain fatty acids were shown. Finally, by considering the use of routine neonatal screening data, this study represents a first step in identifying prognostic markers for cCMV outcome.

3.5. CONFLICTS OF INTEREST

The authors declare no conflict of interest.

3.6. ACKNOWLEDGEMENTS

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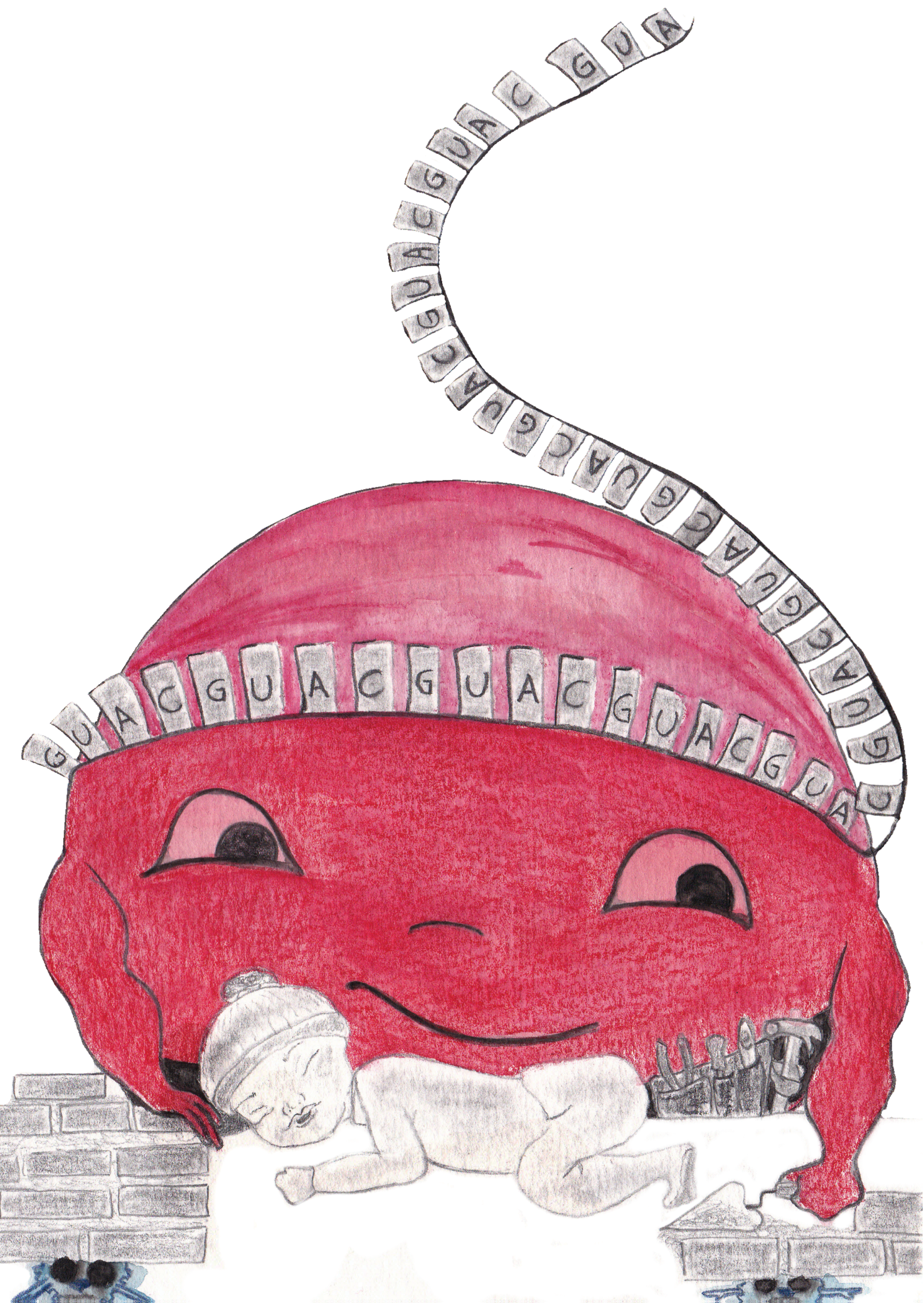
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CHAPTER 4

IMPACT OF CONGENITAL CYTOMEGALOVIRUS INFECTION ON TRANSCRIPTOMES FROM ARCHIVED DRIED BLOOD SPOTS IN RELATION TO LONG-TERM CLINICAL OUTCOME

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ABSTRACT

Congenital Cytomegalovirus infection (cCMV) is the leading infection in determining permanent long-term impairments (LTI), and its pathogenesis is largely unknown due to the complex interplay between viral, maternal, placental, and child factors. The cellular activity, considered to be the result of the response to exogenous and endogenous factors, is captured by the determination of gene expression profiles. In this study, we determined whole blood transcriptomes in relation to cCMV, CMV viral load and LTI development at 6 years of age by using RNA isolated from neonatal dried blood spots (DBS) stored at room temperature for 8 years. As DBS were assumed to mainly reflect the neonatal immune system, particular attention was given to the immune pathways using the global test. Additionally, differential expression of individual genes was performed using the voom/limma function packages. We demonstrated feasibility of RNA sequencing from archived neonatal DBS of children with cCMV, and non-infected controls, in relation to LTI and CMV viral load. Despite the lack of statistical power to detect individual genes differences, pathway analysis suggested the involvement of innate immune response with higher CMV viral loads, and of anti-inflammatory markers in infected children that did not develop LTI. Finally, the T cell exhaustion observed in infected neonates, in particular with higher viral load, did not correlate with LTI, therefore other mechanisms are likely to be involved in the long-term immune dysfunction. Despite these data demonstrate limitation in determining prognostic markers for LTI by means of transcriptome analysis, this exploratory study represents a first step in unraveling the pathogenesis of cCMV, and the aforementioned pathways certainly merit further evaluation.

4.1. INTRODUCTION

Human Cytomegalovirus (CMV) is one of the most common causes of congenital viral infection, leading to a significant number of children with permanent disabilities. The overall birth prevalence of congenital CMV infection (cCMV) in industrialized countries is between 0.6% and 0.7% (1, 2). Among the congenitally infected infants, 12.7% are estimated to have symptoms at birth, ranging from mild, such as petechiae, to severe, such as microcephaly (1, 2). An estimated 40-58% of these symptomatic children develop permanent long-term disabilities, such as hearing loss, cognitive and motor developmental delay (1). Although symptomatic neonates have a considerable risk to develop permanent long-term impairments (LTI), approximately 13% of the asymptomatic children will also develop permanent LTI (1). Despite the current insights into the clinical outcome of cCMV, the multifactorial process that determines whether a child is symptomatic at birth or will develop LTI is largely unknown.

The control of cCMV, and cCMV-related disease, may be the result of a complex interaction between viral, maternal, placental, fetal and child factors (3). The clinical impact of cCMV has mainly been evaluated in relation to maternal factors, such as the CMV immune status before pregnancy or the time of vertical transmission. The vertical transmission rate is higher among women without prior CMV infection than among previously exposed women (2), indicating that pre-existing immunity can be protective. Vertical transmission occurring in the first 20 weeks of pregnancy leads to a worse clinical outcome than transmission occurring later in pregnancy (4, 5). The latter is probably related to an increased susceptibility to infection due to fetal organogenesis, and a still developing fetal immune system. Although the pathogenesis of LTI is poorly understood, the fetal and neonatal immune system likely play an important role in controlling the infection, thereby influencing LTI development (3). Several studies have demonstrated a CMV-specific adaptive immune response in congenitally infected children, such as γδ and γδ T cells or B cells (6-10), as well as an innate immune response (11, 12). However, only few studies have evaluated these responses in relation to clinical outcome at birth, whereas the majority has not done so in relation to LTI development. An increase of NK cells was observed in congenitally infected children, and their frequency was higher in those who were symptomatic at birth (11). In proteomic studies, an increase of macrophage-derived cytokines was observed in congenitally infected children, whereas an increase of γ-defensin was observed in those who were asymptomatic at birth (12). Moreover, the cytokine profile of congenitally infected children, both asymptomatic and symptomatic, was different from that of their mothers with primary infection (13).

The gene expression profile captures a snapshot of the cellular activity which is the result of the response to genetic, environmental and epigenetic factors (14). After having established, through forensic studies, that reliable RNAs can be extracted from dried stains, a considerable amount of studies focused on neonatal dried blood spots (DBS) because they represent an important archived, and readily accessible specimen to study factors of disease development. Indeed, DBS are usually collected at birth for the screening of rare genetic metabolic disorders, and are stored for several years (15). Previous studies have shown that quantitative RNA measurements, either with microarrays or RNA-seq, can be performed on neonatal DBS stored at room temperature for up to

9 years (14, 16-18). Additionally, since the transcriptional profiles of RNA derived from DBS in mice, stored for several months at room temperature, correlated with those from fresh whole blood (19), we assumed this may also be the case in humans. The transcriptome varies according to the cell types studied, and certain RNA markers are tissue-specific. Tissue-specific RNA molecules have been successfully extracted from blood and saliva stains, dried at room temperature for up to 16 years, and used for genome-wide expression analysis (20, 21). Since DBS are produced by spotting whole blood on filter paper, they were assumed to mainly reflect the neonatal immune system.

The aim of this exploratory study was to evaluate the feasibility of transcriptome analysis from archived neonatal DBS in relation to cCMV and LTI development. In particular, we wanted to determine whether the neonatal immune system at birth may be a determinant of LTI development at 6 years of age. This would provide insights into the immune regulation of cCMV, and, by identifying prognostic markers for clinical outcome, could provide the means to introduce the long-debated newborn screening program for CMV in DBS by defining subgroups of infants that would benefit from clinical and audiological follow-up, and possibly antiviral treatment (22). Our investigations revealed that transcriptome analysis of RNA from neonatal DBS stored at room temperature for 8 years of a nation-wide retrospective cohort of children with cCMV and controls is possible, and could potentially be used to unravel the pathogenesis of cCMV and CMV-related disease.

4.2. MATERIALS AND METHODS

4.2.1. Study population and clinical data

A previously described nationwide, retrospective cohort was used in this study (23). The cohort was derived from a total group of 31,484 children, born in 2008 in the Netherlands, which was retrospectively tested for cCMV by PCR of CMV DNA in neonatal DBS at five years of age. In total, 156 children (0.5%) were diagnosed with cCMV. Clinical data were retrieved from 133 congenitally CMV-infected children and from 274 non-infected children. Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or purpura, hypotonia, abnormal laboratory findings (elevated liver transaminases, hyperbilirubinemia, neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domain (hearing, visual, neurological, motor, cognitive and speech-language). The cCMV associated LTI in the original cohort has been described in detail (24). In brief, hearing impairment was defined as sensorineural hearing loss ≥ 40 dB; visual impairment was defined as a visual acuity below 0.3; neurological impairment included cerebral palsy, epilepsy, microcephaly, autism spectrum disorder and ADHD; motor developmental delay was based upon the physical therapist's report and if available on a score below the fifth centile in the Movement Assessment Battery for Children; cognitive developmental delay was defined as an intelligence quotient less than or equal to 70 if this was tested, or it was based on a diagnosis by a medical specialist; speech and language development were assessed by the speech therapist or speech and hearing centre. Additionally, the severity of the LTI was assessed by accumulating the number of domains affected and indicated as the presence of LTI in two or more domains. Since in this cohort

maternal seroimmunity to CMV before birth was unknown, it was assumed that cCMV infection could have resulted from either maternal primary or secondary infection. Due to the retrospective design of the study, there was no standardized clinical and laboratory assessment performed at birth. Therefore, we cannot exclude the possibility that we might have misclassified some newborns without clinically apparent disease or with mild and transient symptoms in the asymptomatic group. However, because of the Dutch child health care system, the chance of having missed major signs or symptoms can be considered negligible (23, 24).

For the study presented in this article, DBS were selected based on the clinical outcome of the infants, with a total of 6 CMV-negative without any clinical signs, 6 CMV-positive with LTI and 6 CMV-positive without LTI. This study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and all the parents of the children included have given written informed consent for the use of clinical data and DBS.

4.2.2. DNA extraction from DBS and qPCR of CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment (RIVM), a second confirmatory PCR was performed at the Leiden University Medical Center (LUMC) (23). For this purpose, DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (25). For each test, one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR, as described previously (26, 27), on the CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate, and the CMV viral load was expressed in IU/ml.

4.2.3. RNA extraction from DBS

One full DBS was punched using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). RNA was extracted from DBS by using the NucleoSpin miRNA kit (Macherey-Negel, Duren, Germany), according to the manufacturer's instructions with a minor modification. This included pre-incubating the DBS with 300 µl of lysis buffer ML for 30 min at 37°C with agitation (1000 rpm) (28). The supernatant was transferred to the NucleoSpin Filter, and the procedure was carried out according to the manufacturer's instruction. Small and large RNAs were purified in one fraction, without separation of small RNAs, and a DNase treatment was used to reduce DNA contamination. The RNA was eluted in 50 µl of RNase-free H_2O, and RNA integrity was assessed using the RNA Nano 6000 Assay Kit on the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The RNA concentration was measured using a Qubit 2.0 flurometer (Life Technologies, CA, USA).

4.2.4. Library preparation and sequencing

An average amount of 185 ng of RNA was used as input material for library preparation. Sequencing libraries were generated using the TruSeq Stranded Total RNA Sample preparation kit for Illumina (Illumina, Inc., San Diego, CA, USA) following the manufacturer's recommendations, and index

codes were added to attribute sequences to each sample. Briefly, rRNA was depleted from total RNA using rRNA removal magnetic beads (RRB). The remaining RNA was purified using RNAClean XP magnetic beads. As the RNA samples from DBS were already fragmented, the fragmentation step was skipped in order to avoid over-fragmentation. First strand cDNA was synthetized using random hexamer primers and SuperScript II reverse transcriptase. Second strand synthesis was performed using the polymerase provided with the kit. After adenylation of the 3' end of the blunt-ended DNA fragments, the RNA index adapters were ligated, and PCR was carried out using the PCR master mix and primer cocktail provided by Illumina to amplify the DNA in the library that had adapter molecules on both ends. Library quality was assessed using the DNA 1000 Assay kit for the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA), and the DNA amount was measured using a Qubit 2.0 flurometer. Clustering of the index-coded samples was performed using the Illumina TruSeq PE Cluster Kit v3 (cBot-HS) according to the manufacturer's instructions. After cluster generation, the libraries were sequenced on the Illumina HiSeq 2000 platform (6 samples per lane), and 76 base paired-end reads were generated for the first batch (n=6, 2 of each group) and 50 base paired-end reads for the second batch (n=12, 4 of each group). All 76 base reads were trimmed to 50 bases to allow for uniform subsequent analysis across all samples, and the batch effect was accounted for in downstream analysis. Due to lack of resources it was not possible to sequence the whole cohort.

4.2.5. Read mapping to the reference genome

Sequence files were generated in FASTQ format, and all RNA sequence files were processed using the BIOPET Gentrap pipeline version 0.7 developed at the LUMC (http://biopet-docs.readthedocs.io/en/latest/releasenotes/release_notes_0.7.0/). The BIOPET Gentrap pipeline consists of FASTQ pre-processing (including quality control, quality trimming and adapter clipping), RNA-seq alignment, read and base quantification. FastQC version 0.11.2 was used for raw read quality control. Low quality read trimming was done using sickle version 1.33 with default settings. Cutadapt version 1.9.1 with default settings was used for adapter clipping based on the detected adapter sequences by FastQC toolkit. RNA-seq reads were aligned against human reference genome GRCh38 using RNA-seq aligner GSNAP version 2014-12-23 with settings "--npaths 1 --quiet-if-excessive". Ensembl human genome annotation version 87 was used for raw read counting. The gene read quantification step was performed using htseq-count version 0.6.1p1 with the setting "--stranded=reverse".

4.2.6. Differential expression analysis: individual genes

We identified significant gene expression differences between congenitally infected children (n=12) and controls (n=6), as well as between congenitally infected children that developed LTI (n=6) and congenitally infected children that did not develop LTI (n=6). Moreover, we also assessed gene expression differences in relation to logarithm of CMV viral load treated as continuous variable. Genes with low fragment counts were removed by requiring at least 2 fragments per million of aligned fragments to be observed in at least 2 samples. Library size normalization factors were obtained with the trimmed mean of M-values (TMM) method (29). Linear modelling using Bioconductor/R package 'limma' (30) was performed on read counts transformed to log-CPM values. Observational-

level weights obtained from the voom function were used to model mean-variance relationship. All three analyses were corrected for the batch effect in the design matrix. Multiple testing correction using false discovery rate control of Benjamini and Hochberg was performed at the threshold of 0.05.

4.2.7. Differential expression analysis: pathways

The Bioconductor/R package 'global test' designed by J. Goeman was used to evaluated differences in expression profiles of gene sets between the different groups (31). These were a group of congenitally infected children (n=12) and a group of controls (n=6). Within the group of congenitally infected children, those that developed LTI (n=6) and those that did not develop LTI (n=6). An additional analysis was performed to find gene set expression profiles dependent on CMV viral load as continuous variable. This method has been shown to have more power to detect gene sets with small effect size (29, 32, 33). We selected a limited number of candidate gene sets (pathways) for use in the global test, before inspecting the data using the QuickGO browser (34). The pathways were selected based on their putative role in the etiology of the disease. An additional selection criterion was the specimen, i.e. DBS, which derives from whole blood and therefore mainly reflects the neonatal immune system. These pathways were T-, B-, and NK-cell activation, innate immune response, and inflammatory response with its regulation. Each pathway contained from 17 to 435 genes. This analysis was performed on the voom-transformed data. Due to the exploratory nature of this study, and to the limited number of selected pathways, no multiple testing correction was applied.

Finally, an additional immune pathway that has emerged as one of the possible players in limiting the immune response during cCMV is the T cell exhaustion (7). However, this does not exist yet as a pathway in the QuickGo browser. Therefore, based on the transcriptional definition of exhaustion previously described (7, 35), and on our available data, a set of exhaustion genes was selected. An independent sample t-test was used to evaluate the difference in the square root of the reads per million (RPM) between the different categories. CMV+ vs CMV-, CMV+ without LTI vs CMV+ with LTI, CMV+ low load vs CMV+ high load. In the latter, the infected group was split in two according to the median log2 viral load measured in DBS which was 10.2, namely low (< 10.2) and high (≥ 10.2) viral load groups. However, p-values were not reported because this analysis had the sole purpose of illustrating trends.

4.3. RESULTS

4.3.1. Study population and clinical data

The clinical data of the congenitally infected children included in this study, as well as of the non-infected controls, are listed in Table 1. A total of 12 children with cCMV, and 6 without cCMV, were included in order to assess the gene expression profile in relation to cCMV. Additionally, the 12 children with cCMV were selected in order to assess differences in gene expression in relation to LTI development. For this purpose, 6 infected children were selected, who did not have any symptoms at birth nor LTI at six years of age, whereas the other 6 had LTI in one or more of the following

Table 1. Study population and clinical outcome

	cCMV with LTI[1] n = 6	cCMV no LTI[2] n = 6	No cCMV[3] n = 6
Gender			
Male	4	3	3
Female	2	3	3
Gestational age (weeks)[4]	39 (36-40)	40 (37-41)	41 (37-41)
Birth weight (g)[4]	3040 (1890-4040)	3340 (2760-4240)	3298 (3070-4360)
CMV viral load[5]	3.1 (2.43-4.97)	3.1 (2.18-4.30)	-
Long term impairment			
Hearing impairment[6]	0	0	0
Visual impairment[7]	0	0	0
Neurological impairment[8]	3	0	0
Motor impairment[9]	6	0	0
Cognitive impairment[10]	4	0	0
Speech/language problem[11]	4	0	0
More than one impairment[12]	5	0	0

[1] Congenitally infected children that develop LTI, 5 out of 6 had symptoms at birth including prematurity (n=1), dysmaturity (n=1), microcephaly (n=3); [2] Congenitally infected children that did not develop LTI, none of them had symptoms at birth; [3] Non-infected controls, none of them had symptoms at birth nor LTI; [4] Values are medians with minimum and maximum; [5] CMV viral load measured on DBS, values are log (IU/ml) medians with minimum and maximum; [6] Sensorineural hearing loss ≥ 40 decibels; [7] Optic nerve atrophy or cortical visual impairment; [8] Cerebral palsy (n=1), epilepsy (n=1), microcephaly (n=1), autism (n=2), ADHD (n=1); [9] Motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder (n=6); [10] Cognitive impairment based on test or diagnosis (n=4); [11] Language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder (n=4); [12] Impairment in two or more domains of impairment: hearing, visual, neurologic, motor, cognitive, and speech-language.

domains of impairment: neurological, motor, cognitive and speech/language (Table 1). Five children out of those who developed LTI also had symptoms at birth. Importantly, none of the children in the control group had symptoms at birth nor developed LTI. Given the diversity of the specific symptoms at birth and impairments at the age of six, the subjects were selected in order to have a similar proportion of male and female across the groups. In this way, the influence of gender in the gene expression analysis was limited.

4.3.2. Library preparation and sequencing statistics

The average number of RNA-seq read pairs per sample was 38.5 million ± 4.8 million, with 38.9 million ± 5.4 million for the CMV- samples and 38.4 million ± 4.7 million for the CMV+ samples. Within the CMV+ samples, those without LTI generated 37.6 million ± 5.9 million paired-end reads, and those with LTI generated 39.1 million ± 3.6 million paired-end reads. The mean RNA fragment size was 285 ± 8 bp, and the mean DNA fragment size was 165 ± 8 bp. On average, 92.25 % of bases exceeded Q30. The detailed information per sample is shown in Table 2.

Table 2. RNA-seq data per individual

ID[1]	cCMV[2]	Gender[3]	LTI[4]	Input RNA (ng)[5]	RNA fragment size (bp)	DNA fragment size (bp)	Total number of read pairs[6]	Total bases[7]	Raw bases Q10+[8]	Raw bases Q20+[9]	Raw bases Q30+[10]
1	CMV-	m	no	160	274	154	36559606	3655960600	3623399872 (99.1%)	3582227412 (98.0%)	3398394726 (93.0%)
2	CMV-	f	no	200	286	166	33157384	3315738400	3286143611 (99.1%)	3247394425 (97.9%)	3076116879 (92.8%)
3	CMV+	f	no	200	278	158	29540831	2954083100	2927528373 (99.1%)	2892987291 (97.9%)	2740849118 (92.8%)
4	CMV+	f	no	200	282	162	31300956	3130095600	3102402121 (99.1%)	3066377738 (98.0%)	2905730026 (92.8%)
5	CMV+	f	yes	120	281	161	33323826	3332382600	3302791747 (99.1%)	3265370156 (98.0%)	3096491305 (92.9%)
6	CMV+	m	yes	200	282	162	39311864	3931186400	3897307546 (99.1%)	3853368879 (98.0%)	3657532714 (93.0%)
7	CMV-	f	no	200	285	165	45571592	4557159200	4499488134 (98.7%)	4432680661 (97.3%)	4219189147 (92.6%)
8	CMV+	f	yes	200	278	158	42119123	4211912300	4158027595 (98.7%)	4097388499 (97.3%)	3904204865 (92.7%)
9	CMV+	m	no	140	286	166	43750889	4375088900	4319612015 (98.7%)	4258366454 (97.3%)	4066273713 (92.9%)
10	CMV-	f	no	200	282	162	35039051	3503905100	3463528125 (98.8%)	3416670543 (97.5%)	3265849570 (93.2%)
11	CMV+	m	yes	200	289	169	40100801	4010080100	3951127179 (98.5%)	3878646166 (96.7%)	3587779279 (89.5%)
12	CMV+	m	no	200	298	178	41322383	4132238300	4081237277 (98.8%)	4023236869 (97.4%)	3838652400 (92.9%)
13	CMV-	m	no	200	287	167	45568773	4556877300	4486847437 (98.5%)	4416030829 (96.9%)	4204748423 (92.3%)
14	CMV+	m	yes	200	289	169	36627608	3662760800	3618784465 (98.8%)	3566791324 (97.4%)	3397972122 (92.8%)
15	CMV+	f	no	200	302	182	41894051	4189405100	4140976514 (98.8%)	4084677610 (97.5%)	3904733403 (93.2%)
16	CMV-	m	no	200	271	151	37242565	3724256500	3638553568 (97.7%)	3565521893 (95.7%)	3317637210 (89.1%)
17	CMV+	m	yes	140	290	170	43235245	4323524500	4228806228 (97.8%)	4149633476 (96.0%)	3947504971 (91.3%)
18	CMV+	m	no	170	282	162	38075664	3807566400	3753496088 (98.6%)	3696000143 (97.1%)	3467378795 (91.1%)

[1] ID child identification number; [2] cCMV, congenital Cytomegalovirus infection; CMV+, congenitally infected children; CMV-, non-infected controls; [3] f, female; m male; [4] LTI, long-term impairment at 6 years of age; [5] Input RNA (ng) the amount of RNA used as input material for library preparation; [6] Total number of paired-end reads, total number of paired-end reads that passed Illumina filter generated per sample; [7] Total bases, total number of bases generated per sample; [8] Raw bases Q10+, base calls with quality Q-scores of Q10+ (Q10 or higher) have an error probability of 0.1 (1 in 10) or less; [9] Raw bases Q20+, base calls with Q20+ have an error probability of 0.01 (1 in 100) or less; [10] Raw bases Q30+, base calls with Q30+ have an error probability of 0.001 (1 in 1,000) or less.

4.3.3. Differential expression: individual genes

Next, we determined whether any other gene could be associated with cCMV, LTI development at 6 years of age or CMV viral load. After low count features removal, ~25% of counts aligned on features and 18360 different genes were used in gene expression analysis. The R package LIMMA was used for the assessment of differential expression of individual genes between congenitally infected children (n=12) and non-infected controls (n=6). No statistically significant differences in gene expression were observed between the groups. We next assessed gene expression differences in relation to cCMV clinical outcome by comparing congenitally infected children that developed LTI at six years of age (n=6) to congenitally infected children that did not develop LTI (n=6). This analysis did not reveal any statistically significant differences between the groups. Finally, the differences in gene expression were assessed in relation to the logarithm of CMV viral load as continuous variable, and no statistically significant differences were observed.

4.3.4. Differential expression: pathways

In order to evaluate whether different biological mechanisms may underlie different clinical outcomes, a global test was performed on manually pre-selected pathways based on their putative role in the etiology of cCMV disease. The selected pathways for T-, B-, and NK-cell activation, innate immune response, and inflammatory response were assessed in relation to cCMV, LTI development at 6 years of age and CMV viral load. The results are shown in Table 3. This analysis revealed trend significant results in relation to CMV viral load and LTI development. In particular, the innate immune response (p=0.046, Fig 1) and the NK-cell activation (p=0.086) may be associated to CMV viral load; whereas the regulation of inflammatory response (p=0.077, Fig 2) to LTI development. In all cases, a small number of genes appeared to be responsible for these trends. Several antiviral genes were positively associated with CMV viral load, i.e. ISG15 and RSAD2, whereas the anti-inflammatory cytokine IL-4 was associated with the congenitally infected children that did not develop LTI.

Table 3. Global Test analysis

	cCMV[1]	LTI[2]	CMV viral load[3]
Pathways	**p-values**		
Innate immune response (435 genes)	0.706[4]	0.432	0.046
T cell activation (51 genes)	0.375	0.203	0.195
B cell activation (30 genes)	0.367	0.125	0.254
NK cell activation (17 genes)	0.717	0.499	0.086
Inflammatory response (381 genes)	0.725	0.341	0.232
Regulation of inflammatory response (68 genes)	0.577	0.077	0.567
Negative regulation of inflammatory response (78 genes)	0.633	0.339	0.133
Positive regulation of inflammatory response (74 genes)	0.444	0.652	0.791

[1] Gene sets expression differences between CMV- (n=6) and CMV+ (n=12); [2] Gene sets expression differences between congenitally infected children with LTI (n=6) and without LTI (n=6); [3] Gene sets expression differences according to CMV viral load (continuous variable).

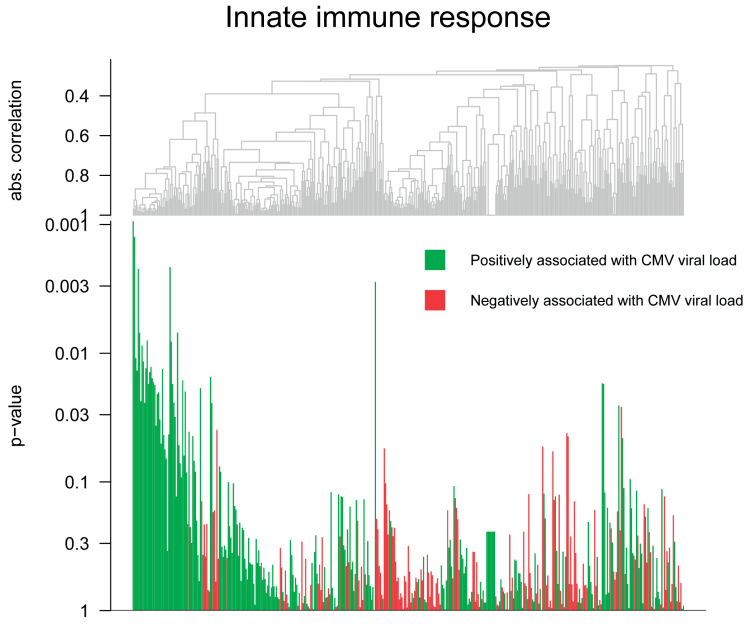

Figure 1. Global test: Innate immune response. Innate immune response in relation to CMV viral load as continuous variable measured on DBS, p=0.046. The gene names of x-axes are provided in supplementary S1 Table.

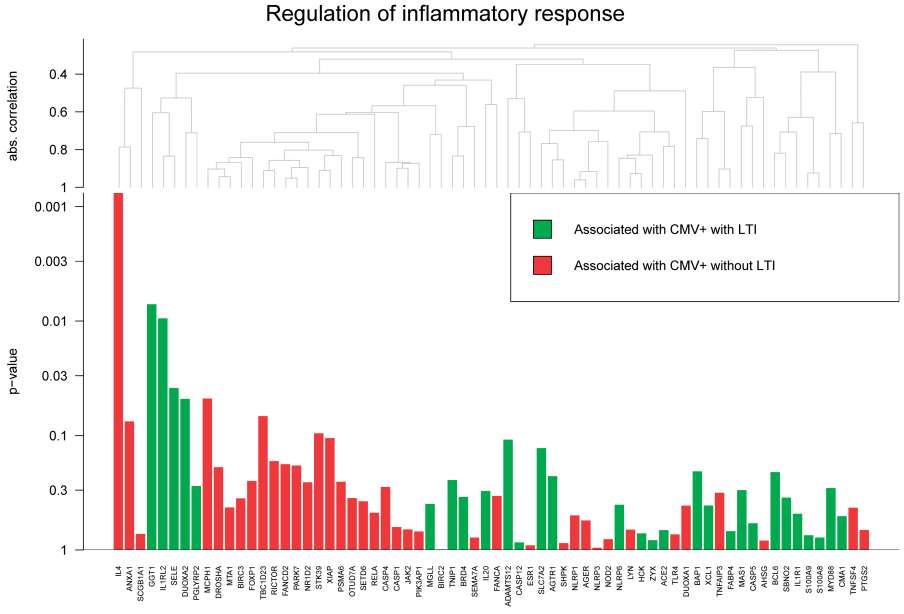

Figure 2. Global test: Regulation of inflammatory response. Regulation of inflammatory response in congenitally infected children that developed LTI at 6 years of age (n=6) and in congenitally children that did not develop LTI (n=6), p=0.077.

Finally, as previously shown by others, one of the possible mechanisms limiting the T cell response to CMV during early life is considered to be T cell exhaustion (7). Therefore, we wondered whether the same phenomenon could be observed in our cohort when comparing the CMV- group (n=6) to the CMV+ group (n=12). Additionally, this pathway was assessed in relation to CMV viral load and development of LTI at 6 years of age. For this purpose, based on the transcriptional definition of exhaustion previously described (7, 35), as well as on our available data, a set of genes was selected and reported in Table 4. Of these genes, the RPM were reported for each comparison in order to observe the trend to be further explored. A trend of increased expression of differentiation markers, mainly CD57 and transcription factor T-bet, and of increased effector markers, primarily IFN-γand granzyme, was observed in the CMV+ group compared to the CMV- group (Fig 3 A-D). Furthermore, a trend of increased expression of inhibitory markers, mainly PD-1 and LAG-3, was observed in the CMV+ group (Fig 3 A-D). Next, the CMV+ group was split in two groups according to the median log2 viral load measured in DBS which was 10.2, namely low and high viral load groups. Comparing the group with high viral load to the one with low viral load, the aforementioned observed trends relative to differentiation, effector and inhibitory markers were more pronounced than when comparing CMV+ to CMV-. Finally, when comparing the cCMV+ group that developed LTI to those who did not, no striking trends were observed (Fig 3 A-D).

Table 4. T cell markers

T cell markers
Differentiation and effectors[1]
IFNγ
IL-2
MIP-1β
TNF-α
Granzyme B
Perforin 1
CCR5
CD57
Transcription factors[2]
T-bet
Blimp-1
Inhibitory receptors
PD-1
LAG-3
FAS-L
Inhibitory cytokines
IL-10
TGF-β

[1] Markers defining a differentiation phenotype that leads to a functional response; [2] Key transcription factors for T cell differentiation and exhaustion

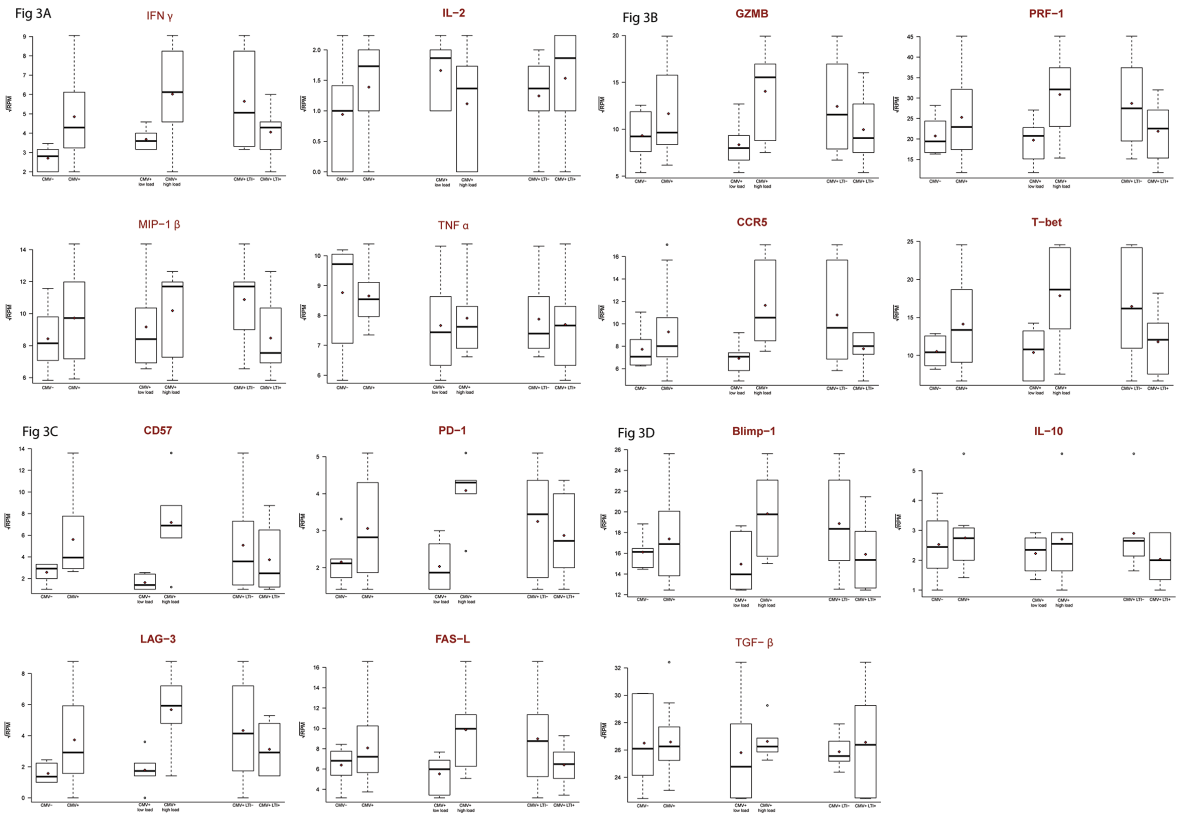

Figure 3. A-D. T cell exhaustion. T cell markers identifying the exhaustion phenotype in relation to cCMV, CMV viral load and LTI development at 6 years of age. *CMV-*, non-infected controls (n=6); *CMV+*, congenitally infected children (n=12); *CMV+ low load*, congenitally infected children with log2 CMV viral load below the median measured in DBS which was 10.2 (n=6); *CMV+ high load*, congenitally infected children with log2 CMV viral load equal to or above the median measured in DBS (n=6); *CMV+ LTI-*, congenitally infected children that did not develop LTI (n=6); *CMV+ LTI+*, congenitally infected children that developed LTI (n=6). Boxplot: *bold line*, median of square root of RPM; *red dot*, mean of square root of RPM.

4.4. DISCUSSION

This study aimed to evaluate whether transcriptome analysis by next generation RNA sequencing on DBS derived from a retrospective nation-wide cohort of children with cCMV and controls is feasible, and whether useful insights could be obtained on the etiology of different cCMV outcomes. This would allow the identification of potential biomarkers for long-term outcome, which could provide the means to introduce the long-debated newborn screening program for cCMV in DBS (22). Indeed, this would define subgroups of children benefitting from clinical, audiological follow-up, and possibly antiviral treatment.

The global test for differential expression of gene sets revealed, although only with trend significant results, an important feature of cCMV in relation to whole blood transcriptome, i.e. CMV viral load is the main factor to influence the pre-selected immune pathways, whereas CMV disease seems to be secondary. In our study, numerous antiviral genes were positively associated with CMV viral load, suggesting the involvement of the innate immune system in response to cCMV in the newborns, in particular with higher viral loads. The fact that no striking differences were observed when comparing CMV+ to CMV-, suggests that the high viral load is the main initiator of this expression pattern. Therefore, the presence of neonates with low viral load in the CMV+ group may have diluted the differences between CMV+ and CMV-. Congenitally infected children excrete CMV for several years after birth, whereas in adults this lasts only several months (36, 37), suggesting a deficient cell-mediated immune response in early life (38). Therefore, it is tempting to speculate that the activation of the innate immunity in the fetus may have an important role in controlling cCMV, however this is difficult to determine. One of the possible mechanism for this limited T cell response to CMV during early life is considered to be T cell exhaustion (7). In our cohort, also the exhaustion pathway was more pronounced in the high viral load group compared to the low viral load group, with PD-1 being the marker influenced the most, as previously shown (7). Therefore, also in this case the difference in exhaustion pathway between CMV+ and CMV- could have been diluted because of the presence of low viral load individuals in the CMV+ group. However, the exhaustion pathway analysis needs further confirmation as we only reported expression trends. T cell exhaustion is characterized by loss of T cell functions, and is induced by persistent infections (7, 35). Primary CMV infection induces functional T cell exhaustion in both adults and fetuses, though considerably more pronounced in the latter. As this phenomenon is associated with prolonged exposure with higher viral loads, the high viral loads reported in fetuses may be the cause of this effect (39-41). The exhaustion may contribute to the prolonged CMV viral excretion in the children (7). The influence of viral load in the immune responses has been shown before, both in humans and in the murine models of CMV infection. Here, the degree of CMV-specific memory CD8 T cells accumulation, as well as the phenotypic T cell profile, was influenced by the viral load (42, 43). However, the role of CMV viral load in the clinical outcome still remains controversial. Some studies have correlated CMV viral load, measured in blood, with clinical outcome (44, 45), whereas others have not (46-48). The neonatal viral load may differ depending on the trimester of vertical transmission, or whether it was a primary maternal infection. Indeed, earlier infections may lead to a more extensive cCMV. However, in our cohort this is impossible to establish (49). Additionally,

CMV viral load in whole blood may not correlate to CMV loads in other neonatal compartments, and therefore may not fully reflect viral replication in all affected organs and tissues.

The molecular mechanisms of LTI development are largely unknown, though the late-onset hearing loss is believed to be the result of a chronic productive infection throughout childhood (50, 51). In this context, a long-term dysfunctional immune response seems plausible, although it cannot be excluded that such dysfunction leads to a parallel uncontrolled inflammation that contributes to tissue damage. In studies of characterization of tissue damage in fetuses of 20-21 weeks of gestation with cCMV, an association between the degree of tissue damage in the brain, as well as in the inner ear, with viral load, inflammatory response and placental functionality was shown (52, 53). A dysfunctional immune response that leads to uncontrolled viral replication, and immune-mediated damage was suggested. Therefore, a similar pathogenesis may be assumed when such infection becomes chronic. The exhaustion pathway that was found in congenitally infected children, especially those with higher CMV viral load, did not seem to correlate to clinical outcome at 6 years of age. This suggests that other mechanisms are involved in the long-term immune dysfunction. In our cohort, when comparing congenitally infected children that developed LTI to those infected who did not, a role for the regulation of inflammatory responses seemed to partially contribute. Anti-inflammatory markers, such as the cytokine IL-4, were associated with congenitally infected children that did not develop LTI. The success of an immune response is the result of a balance between effector and regulatory mechanisms, therefore, the potential protective effect of IL-4 in those infected children that did not develop LTI may lie in its anti-inflammatory property. Interestingly, in a cohort of healthy CMV infected individuals, the CD4 T-cell response associated with a protective immunity involved cytokine production of IFN, and/or IL-17, in association with IL-4 (54). Similarly to IL-10, IL-4 has been shown to possess the capacity of down-regulating the production of pro-inflammatory mediators by microglia, both in humans and in mice (55-57), and its neuroprotective effect was associated with downregulation of brain inflammation in mice (58). When studying the regulation of the inflammatory response in children with cCMV and compare the group with LTI to that without LTI, we have to be aware that there may be other perinatal factors influencing the inflammatory pattern in DBS. Although we cannot fully exclude a role for non-cCMV related perinatal factors, there was no bacterial amniotic infection or neonatal sepsis in all children included in this study.

Several reasons may have contributed to the fact that we did not find a strong impact of cCMV on whole blood transcriptomes from DBS. First of all, one of the groups of congenitally infected children did not have symptoms at birth nor LTI, which is the case in most children with cCMV, and the clinical signs of symptoms associated with LTI are very diverse. Second of all, in our cohort, the fetal infection may have been the result of a primary or secondary CMV infection in the mother, and may have taken place at any time during pregnancy, especially in the asymptomatic children. Third of all, the small sample size of the groups may have led to a lack of power both in the gene expression analysis of individual genes, as well as in the pathway analysis. Lastly, the RNA degradation on these specimens, due to e.g. ribonucleases, pH, humidity or UV light, may have contributed to the lack of significant differences among the sample groups. The degradation of RNA from dried stains has been extensively studied in forensic studies for obvious reasons, and several RNAs have

been extracted from numerous conditions (59-64). From these studies, determinants for RNA stability appeared to be the specimen the RNA is extracted from, and the specific RNA molecule analyzed. In the former, the detection limit of blood-specific RNA has been shown to be lower than for other specimens (21). In the latter, some RNAs can be more stable in dried stains than others (21). Secreted RNAs, e.g. in fresh saliva, may be more susceptible to fast degradation by extracellular RNases, and therefore are not to be expected on dried stains (20). Importantly, for those RNAs detected on dried blood stored at room temperature, only few genes have been demonstrated to be differentially expressed during time (20). Therefore, we assumed that those markers detected on DBS in our study were less prone to degradation, and relatively stable for long periods of time. Furthermore, the influence of RNA contamination in the downstream analysis, e.g. from skin cells or external microorganisms, may be considered negligible as the most abundant RNAs species come from the host whole blood (65). Despite the fact that enough data were generated in our study for the downstream analysis, with comparable cDNA fragment size as shown in forensic studies (21, 59-64), we cannot exclude that fresh material may have revealed differences in expression patterns that we could not pick up.

Furthermore, due to the retrospective nature of the study, cCMV diagnosis was carried out by performing PCR of viral DNA on DBS, which in comparison with PCR on urine or saliva has been associated with limited and variable sensitivity (66). Therefore, a negative CMV PCR on DBS does not fully exclude cCMV. However, it is important to note that with the relatively high sensitivity of our CMV PCR on DBS (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (23). Therefore, it is very unlikely that a cCMV positive child ended up in our cCMV negative control group.

To the best of our knowledge, this is the first exploratory study assessing the feasibility of transcriptome sequencing using RNA isolated from archived neonatal DBS of children with cCMV, and non-infected controls, in relation to long-term outcome. Despite the lack of statistical power to detect individual gene expression differences, the pathway analysis suggested a potential differential gene expression in relation to CMV viral load and LTI. Therefore, this study represents a first step in unraveling the pathogenesis of cCMV, and in identifying prognostic markers for cCMV long-term outcome.

4.5. ACKNOWLEDGMENTS

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4.6. AUTHOR CONTRIBUTIONS

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4.7. DATA AVAILABILITY STATEMENT

The dataset underlying this study cannot be shared publicly, as it would violate restrictions imposed by the Medical Ethical Committee of Leiden University Medical Center (LUMC). Specifically, the authors must restrict the full genomic data of the participants of the study in order to protect participant privacy. A minimal underlying data set containing the read counts per gene value is available in the Supporting Information files. Interested and qualified researchers may request access to the full dataset by contacting Eric C.J. Claas, Molecular Biologist of the Medical Microbiology Department of LUMC, E.C.J.Claas@lumc.nl.

4.8. FUNDING

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4.9. COMPETING INTERESTS

We have the following interests: HL is a founder and the chairman of Alacris Theranostics GmbH, Berlin, Germany. There are no patents, products in development or marketed products to declare. This does not alter our adherence to all the PLOS ONE policies on sharing data and materials.

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SUPPORTING DATA

Table S1. Gene names. Gene names of x-axis of Fig 1 concerning the innate immune response in relation to CMV viral load as continuous variable. (XLSX)

Genes	p-value	Genes	p-value	Genes	p-value
ISG15	0,001	IFI16	0,043	CLEC5A	0,142
RSAD2	0,001	SPON2	0,044	PRKD1	0,149
GSDMD	0,002	GPER1	0,044	CLEC10A	0,150
OASL	0,002	TNK1	0,046	TRIM14	0,154
IGHG1	0,003	DHX58	0,050	IGHG4	0,155
PRDM1	0,007	B2M	0,052	AIM2	0,159
OAS3	0,007	IL36RN	0,053	SIGLEC15	0,159
BST2	0,008	CD300LB	0,055	MST1R	0,160
NLRC5	0,008	TRIM21	0,056	CSK	0,160
IFIT3	0,009	NR1H4	0,058	TRIM26	0,164
PML	0,011	SLAMF7	0,062	IL36B	0,165
HERC5	0,012	MX2	0,066	LY86	0,166
IFIH1	0,013	KLRD1	0,069	SIRPB1	0,166
SERPING1	0,013	ELF4	0,072	CFI	0,167
ADAR	0,013	SARM1	0,076	IGHV3OR16-9	0,170
APOL1	0,014	MATK	0,083	IL1RL2	0,172
EIF2AK2	0,014	ZAP70	0,083	PGLYRP2	0,182
JAK3	0,015	APOBEC3D	0,084	SERINC3	0,186
IFIT5	0,016	TBK1	0,092	DEFB116	0,192
ZNF683	0,016	PTK2B	0,095	IFNA6	0,193
MX1	0,017	IRAK1	0,100	SRC	0,195
IFITM3	0,017	FCN1	0,102	HMGB2	0,200
FCGR1A	0,017	TRIM5	0,103	IFNL1	0,200
IRF7	0,017	IFNA21	0,108	FGA	0,202
DDX60	0,017	APCS	0,112	SRPK1	0,205
OAS2	0,018	UBC	0,114	IL27	0,207
APOBEC3F	0,019	SIGLEC14	0,117	TRIM11	0,208
APOBEC3G	0,020	IFNA13	0,120	NLRX1	0,213
IFITM1	0,020	IRF1	0,121	F12	0,213
IFIT1	0,021	APOBEC3C	0,123	MR1	0,215
ZBP1	0,024	IL23A	0,124	FES	0,215
ISG20	0,024	IGHG2	0,125	NFKB1	0,221
IFIT2	0,024	ANXA1	0,126	KLRG1	0,225
TBKBP1	0,024	TRIM15	0,129	CFP	0,225
NLRP2	0,025	IGHA1	0,130	GATA3	0,227
C4B	0,026	BPIFB1	0,130	NLRP3	0,227
ZC3HAV1	0,031	NOD1	0,130	FBXO9	0,230
DDX58	0,033	JCHAIN	0,131	BPIFB3	0,233
OAS1	0,037	NRROS	0,134	IGHV3-23	0,234
DEFB129	0,039	TNK2	0,136	KLRK1	0,238
SLAMF6	0,041	SLAMF1	0,138	TEC	0,238
DEFB114	0,042	VSIG4	0,138	IKBKG	0,243
TRIM25	0,043	FRK	0,141	DEFB105B	0,244

Table S1. (continued)

Genes	p-value	Genes	p-value	Genes	p-value
DEFB106A	0,244	CD84	0,350	APP	0,448
DEFB4B	0,244	DEFB107A	0,352	S100A9	0,449
DEFB103B	0,244	DEFB113	0,356	IFNA10	0,452
DEFB104B	0,244	TRIM4	0,357	TRIM13	0,453
RP11-330H6.5	0,244	CORO1A	0,358	SSC5D	0,454
DEFB103A	0,244	C4A	0,359	MAP3K5	0,456
C4BPA	0,245	GZMM	0,361	PPP1R14B	0,456
CARD9	0,257	IL1RAP	0,368	SH2D1B	0,460
IL23R	0,257	CASP4	0,369	ABL2	0,463
KLRC2	0,260	IFNL2	0,369	IRAK4	0,464
C8B	0,261	SUSD4	0,369	SLPI	0,467
PTK6	0,264	YES1	0,371	SH2D1A	0,474
APOBEC3B	0,266	COLEC12	0,374	TICAM1	0,478
DEFA3	0,266	TRIM56	0,375	TLR10	0,479
SAA1	0,270	C1R	0,380	CYBB	0,482
TRIM62	0,272	C5	0,381	FER	0,488
SIRT2	0,273	UNC93B1	0,382	MEFV	0,496
C1QBP	0,278	CD6	0,383	DEFB133	0,497
MB21D1	0,281	DEFB134	0,383	RNF135	0,498
SMPDL3B	0,283	MASP2	0,390	IPO7	0,500
TRIM28	0,283	DEFB115	0,391	DEFA1	0,503
CACTIN	0,288	CAPZA1	0,396	TREM1	0,508
LGALS3	0,290	KRT16	0,396	TREML1	0,508
IFNA5	0,292	ANKRD17	0,397	MAP4K2	0,511
APOBEC3A	0,295	PGLYRP4	0,397	TLR3	0,511
HMGB1	0,299	C9	0,401	MIF	0,515
CHID1	0,303	PGLYRP3	0,401	PIK3CG	0,515
BLK	0,311	FCN2	0,410	ITCH	0,518
POLR3H	0,313	CYLD	0,417	SRMS	0,519
RNASE7	0,313	CSF1R	0,417	CSF1	0,520
CLEC7A	0,315	IRGM	0,420	DEFB132	0,523
LCK	0,319	IFNL3	0,421	AKAP8	0,527
NCR2	0,324	TRIM35	0,423	C4BPB	0,530
DMBT1	0,324	IGHE	0,423	TLR6	0,531
C1QB	0,324	DEFB125	0,423	TRIM10	0,532
DEFA5	0,326	RPS27A	0,425	NLRP1	0,538
IFITM2	0,327	CD180	0,427	CD1D	0,553
CR2	0,336	OTULIN	0,427	PYCARD	0,554
POLR3D	0,337	PADI4	0,433	TRIL	0,554
C2	0,340	ITK	0,436	TMEM173	0,556
JAK2	0,344	DEFB126	0,439	TLR8	0,560
IFNA14	0,344	FYN	0,440	NLRC4	0,562
DAB2IP	0,345	DEFA4	0,440	TRIM38	0,569
ECSIT	0,349	ATG5	0,440	TRIM8	0,570
AXL	0,349	PIK3CD	0,445	LILRA5	0,571
ADAM15	0,349	FCN3	0,447	POLR3A	0,574

Table S1. (continued)

Genes	p-value	Genes	p-value	Genes	p-value
DEFB105A	0,575	PSTPIP1	0,672	TLR9	0,799
ATG12	0,576	CRISP3	0,677	MBL2	0,804
SYK	0,582	DEFB107B	0,678	BCL10	0,806
C1QC	0,583	ACOD1	0,691	IFNA4	0,809
F2RL1	0,584	VNN1	0,691	DEFB108B	0,814
CRCP	0,588	DEFA6	0,693	CLEC4M	0,814
APOBEC3H	0,590	CLEC4D	0,697	IFNA2	0,815
CD300E	0,591	BPIFA1	0,699	REL	0,817
LGR4	0,595	MASP1	0,701	IRF5	0,819
AGER	0,596	SFTPD	0,703	IGLL1	0,820
C1S	0,597	TIRAP	0,704	NOD2	0,822
CYBA	0,599	FADD	0,704	DEFB106B	0,823
TYK2	0,600	RELB	0,709	DEFB104A	0,826
BMX	0,602	SEC14L1	0,709	IL36A	0,828
CD209	0,610	RIPK2	0,715	DEFB4A	0,837
CLEC4E	0,614	LY9	0,715	TRIM27	0,841
NAIP	0,615	S100A7	0,715	STYK1	0,848
ANKHD1	0,618	TNFAIP8L2	0,718	MYD88	0,848
DEFB131	0,618	C1RL	0,723	DEFB118	0,850
DEFB123	0,618	ZBTB1	0,727	TRIM31	0,851
DEFB119	0,620	IL36G	0,727	NFKB2	0,853
CTD-2313N18.7	0,622	TKFC	0,730	ANG	0,854
DEFB110	0,622	TLR4	0,732	MID2	0,855
PCBP2	0,622	CNPY3	0,735	NLRP2B	0,859
PPARG	0,626	CFB	0,737	C7	0,862
DEFB135	0,629	CR1	0,738	FGR	0,862
AKIRIN2	0,630	TYROBP	0,740	TICAM2	0,863
TRAF3	0,630	CFD	0,744	CAPZA2	0,864
LCN2	0,631	S100A8	0,745	TOLLIP	0,867
BTK	0,634	C8A	0,749	FGB	0,867
MARCO	0,637	DEFB128	0,749	S100A12	0,873
PGLYRP1	0,640	POLR3E	0,750	POLR3F	0,876
IKBKB	0,640	CLU	0,754	POLR3B	0,876
CLEC6A	0,642	TLR7	0,771	TREM2	0,879
DEFB127	0,645	NLRP6	0,775	CHGA	0,884
IFNA8	0,646	ABL1	0,777	IGLL5	0,886
HAVCR2	0,648	CHUK	0,781	IFNW1	0,890
JAK1	0,648	NCF2	0,782	C1QA	0,893
CFH	0,656	IFNA1	0,787	LY96	0,896
IRF3	0,659	SAMHD1	0,788	HMGB3	0,897
TRIM32	0,660	CLEC4A	0,790	HCK	0,897
POLR3G	0,661	CD14	0,791	TLR5	0,899
C6	0,665	CD244	0,794	MALT1	0,903
DDX3X	0,665	IL34	0,794	IFNA7	0,909
CAMP	0,670	TLR2	0,797	TLR1	0,917
DEFB1	0,671	DEFB124	0,798	IFNE	0,917

Table S1. (continued)

Genes	p-value	Genes	p-value	Genes	p-value
NCF1	0,918	CD46	0,942	FCER1G	0,971
S100B	0,919	PTK2	0,944	LBP	0,973
POLR3C	0,920	ARHGEF2	0,947	C8G	0,973
ADARB1	0,925	CD55	0,949	MSRB1	0,978
PTX3	0,934	UBA52	0,953	SRPK2	0,978
NLRP10	0,936	C3	0,954	POLR3K	0,981
IFNA16	0,939	CLEC4C	0,957	IFNA17	0,984
UBB	0,940	DEFB112	0,957	SERINC5	0,985
LYN	0,940	MAVS	0,964	PYDC1	0,991
SDHAF4	0,941	DEFA1B	0,967	DEFB121	0,991

Table S2. Read counts per gene. (XLSX). Avaiable online.

CHAPTER 5

CONGENITAL CYTOMEGALOVIRUS INFECTION: MATERNAL-CHILD HLA-C, HLA-E AND HLA-G AFFECT CLINICAL OUTCOME

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ABSTRACT

Congenital CMV infection (cCMV) is the most common congenital infection causing permanent long-term impairments (LTI). cCMV immunopathogenesis is largely unknown due to the complex interplay between viral, maternal, placental and child factors. In this study, a large retrospective nation-wide cohort of children with cCMV and their mothers was used. HLA-C, HLA-E, and HLA-G were assessed in 96 mother-child pairs in relation to symptoms at birth and LTI at six years of age. The mothers were additionally typed for killer cell immunoglobulin-like receptors. The maternal HLA-G 14 bp deletion/deletion polymorphism was associated with a worse outcome, as the immunomodulation effect of higher proteins levels may induce less CMV control, with a direct impact on placenta and fetus. The absence of maternal HLA-C belonging to the C2 group was associated with symptoms at birth, as activating signals on decidual NK may override inhibitory signals, contributing to a placental pro-inflammatory environment. Here, the increased HLA-E*0101 and HLA-C mismatches, which were associated with symptoms at birth, may enhance maternal allo-reactivity to fetal Ags, and cause sub-optimal viral clearance. Finally, HLA-C non-inherited maternal antigens (NIMAs) were associated to LTI. The tolerance induced in the fetus towards NIMAs may indirectly induce a sub-optimal CMV antiviral response throughout childhood. In light of our findings, the potential role of maternal-child HLA in controlling CMV infection and cCMV-related disease, and the clinical value as predictor for long-term outcome certainly deserve further evaluation.

5.1. INTRODUCTION

Human CMV is one of the most common causes of congenital viral infection, leading to a significant number of children with hearing loss and neurodevelopmental delay. The overall birth prevalence of congenital CMV infection (cCMV) in industrialized countries lies between 0.6 and 0.7% (1, 2). Among the congenitally infected infants, 12.7% are estimated to have symptoms at birth including petechiae, jaundice, hepatosplenomegaly, thrombocytopenia, chorioretinitis, and microcephaly (1, 2). An estimated 40-58% of these symptomatic children will develop long-term impairments (LTI), such as hearing loss, cognitive and motor developmental delay (1). Of the 87.3% asymptomatic infants, around 13.5% will also develop LTI (1). Despite the current insights into the clinical outcome of cCMV, the multifactorial process that determines whether a child will be symptomatic at birth or will develop LTI is still poorly understood.

The vertical transmission rate is higher among women without prior CMV infection than among previously exposed women, suggesting a role for maternal immunity in the risk of vertical transmission (2). Vertical transmission takes place *via* placental infection. Once CMV infects the placenta, the extensive local damage and inflammation lead to placental dysfunction, which in turn can impair fetal development (3). The clinical impact of fetal infection is largely determined by vertical transmission in the first 20 weeks of pregnancy, during which the fetal immune system is still developing (4, 5). Importantly, the fetal and neonatal immune system may also play a role in controlling the infection, thereby influencing LTI development (6). A persistent productive infection may lead to late-onset or progression of sensorineural hearing loss, even though a role of immunopathology cannot be entirely excluded (7, 8). Hence, clinical outcome is the result of a multifactorial process that comprises maternal, placental, fetal, and child factors.

Pregnancy is considered a semi-allograft, in that the fetus may have HLA antigens (Ags) that the mother does not have. At the maternal-fetal interface the majority of trophoblast cells are in direct contact with maternal cells, and several mechanisms are in place to prevent rejection of the fetal semi-allograft. Extravillous trophoblast cells do not express HLA-A, -B, -DR, -DQ and –DP (9), but they do express HLA-C and the non-classical HLA-E and HLA–G. HLA-C and HLA-E prevent maternal NK cell-mediated cytotoxicity through binding with killer cell immunoglobulin-like receptors (KIRs) expressed on decidual NK cells (dNK). dNK cells are the most abundant leukocyte population in the placenta (9), therefore they play a fundamental role in pregnancy. KIR genotypes can be distinguished into two haplotypes. The KIR-A haplotype mainly contains inhibitory receptors, such as KIR2DL1, whereas, the KIR-B haplotype also contains one or more activating receptors, such as KIR2DS1 (10, 11). NK cytotoxicity is controlled through a balance of both activating and inhibitory receptors on dNK cells (12-15). Furthermore, HLA-G modulates the response of different cellular subsets, including dNK, APC, T cells, and B cells (16, 17). The complex maternal-fetal immune cross-talk at the interface creates a tolerogenic niche for the normal development of the fetus, and it differs from the peripheral immune system of both mother and child (18). Indeed, immune cells can be generated locally with a different function than the one acquired at the periphery. For example, CD8 T cells express significantly lower levels of perforin and granzyme-B, and DC are arrested in a tolerogenic state (18, 19). Each pregnancy is characterized by a unique mother-fetus

HLA combination, which may generate different immune effector and regulatory mechanisms (20, 21). Maternal-fetal HLA-C mismatches (mm) induce a shift towards higher levels of effector and regulatory T cells in the decidua (20). In addition, specific combinations of maternal KIRs and fetal HLA-C can induce pregnancy complications (22, 23). Indeed, the HLA-C ligands for KIRs are divided into two groups, the C1 allotype binds inhibitory KIRs whereas the C2 allotype binds both activating and inhibitory KIRs (10, 11). Therefore, while KIR2DL1/HLA-C2 leads to inhibition, KIR2DS1/HLA-C2 leads to activation of dNK (15, 24). The risk of recurrent miscarriage increases in women with KIR-A haplotype when they have fewer C2 genes than the fetus, or when the fetus inherited C2 from the father (18, 25). Indeed, the proper dNK activation is essential to facilitate trophoblast invasion and a successful placentation (26). Furthermore, lower levels of soluble HLA-G (sHLA-G) have been described in the circulation of pregnant women with pre-eclampsia, intrauterine growth retardation, and recurrent spontaneous abortion (27, 28).

With respect to CMV, the role of HLA in the local immune response in the placenta is largely unknown. With regard to HLA-G, higher CMV viral loads in urine of children homozygous for the HLA-G 14 bp deletion were found (29). In addition, higher levels of sHLA-G were shown in serum and amniotic fluid of women with primary CMV infection and in symptomatic neonates (30). The aim of this study was to determine if the combination of maternal and child HLA Ags influences the short- and long-term outcome of cCMV, thus providing new insights into cCMV immune regulation and pathogenesis. Our investigations focused on the role of HLA-C, HLA-E, and HLA-G, which are the only HLA molecules expressed at the placenta.

5.2. MATERIAL AND METHODS

5.2.1. Study population and clinical data

A previously described nationwide, retrospective cohort was used in this study (31, 32). The cohort was derived from a total group of 31,484 children, born in 2008 in the Netherlands, which was retrospectively tested for cCMV by PCR of CMV DNA in neonatal dried blood spot (DBS) at five years of age. In total, 156 children (0.5 %) were diagnosed with cCMV. Clinical data were retrieved from 125 congenitally CMV infected children and from 263 non-infected children. For this study, buccal swabs from 104 children with cCMV and their mothers were obtained for HLA-typing. Two buccal swabs [FLOQSwabs hDNA Free, 20-mm breaking point in 174.5mm long dry tube (COPAN ITALIA SPA, Brescia, Italy)] were retrieved from each individual. Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or purpura, hypotonia, abnormal laboratory findings (elevated liver transaminases, hyperbilirubinemia, neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domain (hearing, visual, neurological, motor, cognitive and speech-language). Because in this cohort maternal seroimmunity to CMV before birth was unknown, it was assumed that cCMV infection could have resulted from either maternal primary or secondary infection. This study was approved by the Medical Ethics Committee of the Leiden University

Medical Center, and all the parents of the children included have given written informed consent in accordance with the Declaration of Helsinki.

5.2.2. DNA extraction from DBS and qPCR of CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment (RIVM), a second confirmatory PCR was performed at the Leiden University Medical Center (LUMC) (31). For this purpose, DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (33). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR, as described previously (34, 35), on the CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate, and the CMV viral load was expressed in IU/ml.

5.2.3. DNA extraction from buccal swabs

DNA was extracted from the buccal swabs by using the QIAamp DNA (blood) mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction, with an additional PBS pre-incubation of 30 minutes at room temperature. Additionally, the pre-incubation fluid from two swabs was applied to one QIAamp spin column, in order to concentrate the DNA. The DNA was eluted in 150µl of Tris-EDTA-4 buffer for further analyses.

5.2.4. HLA and KIR typing

All mothers and children were DNA typed at low resolution for HLA-C locus (14 alleles) using the Reverse Sequence Specific Oligonucleotides PCR technique. For this purpose, a commercially available assay was applied, LIFECODES C SSO Typing kits (Immucor, Norcross, GA, US). Data were analysed using MatchIT software (Immucor-Lifecodes). The HLA-C alleles included in this study were selected based on 5604 randomly selected healthy Dutch blood donors who were previously genotyped (36). This group is considered to be a proper representation of the gene distribution in the general population (37), and the HLA-C frequencies are depicted in Table S1 in Supplementary Material. HLA-C allotypes were divided in two groups, namely HLA-C1 and HLA-C2, based on the presence of SER77ASN80 (C1) and ASN77LYS80 (C2) at position 80 of the α1 domain, as previously described (38).

All mothers and children were typed for the two most common HLA-E functional alleles, HLA-E*01:01 and HLA-E*01:03 (39). Eight different HLA-E proteins have been identified from 26 coding sequences, but the aforementioned ones, due to their functional differences, are the two most commonly analyzed worldwide (39). Due to a non-synonymous mutation these two functional alleles differ in one aa position in codon 107 of the α heavy chain, HLA-E*01:03 encoded proteins show higher cell surface expression and peptide-binding affinity than HLA-E*01:01proteins (40). Determinations of the HLA-E*01:01/01:03 variants (SNP ID rs1264457) were carried out by using the TaqMan® SNP Genotyping Assays (ThermoFisher Scientific, MA, USA) on a ViiA™ 7 Real-Time

PCR System (ThermoFisher Scientific, MA, USA) in a 96 well-plate using a thermocycling profile as follows: 10 min 95°C followed by 40 cycles of 95°C (15 seconds), 60°C (1 min) and finally after cycling 60°C (30 seconds) with 1.5 µl of diluted DNA extract and 8.5 µl of reaction mix. Data were analyzed using ViiA™ 7 Software.

All mothers and children were typed for the HLA-G locus by using a TaqMan assay for the 14-bp insertion/deletion polymorphism in exon 8 of the 3' untranslated region with primers and probes previously described (41). Nine different HLA-G proteins from 28 alleles have been described (42), however, the polymorphism included in this study is one of the most commonly associated with pregnancy (43-47). The TaqMan assay consisted of 1.5µl diluted DNA extract and 15 µl reaction mixture containing: 7.5 µl TaqMan Universal Master Mix II with UNG (ThermoFisher Scientific, MA, USA), 300 nM of forward and reverse primers, 200 nM of HLAG14FAM (insertion) and HLAGdelCY5 (deletion) probes. The PCR was performed on a Light Cycler®96 Detection System (Roche Applied Science, Mannheim, Germany) in a 96 well-plate using a thermocycling profile as follows: 10 min 95°C followed by 50 cycles of 95°C (10 s), 58°C (50 s) and finally after cycling 37°C (30 s). Data were analyzed with Light Cycler®96 Analysis Software 1.1.

All mothers were genotyped at low resolution for KIR receptors. The KIR genotype differs based on the absence of activating KIRs (haplotype AA) or on the presence of different numbers of activating KIRs (haplotype AB or BB). In particular, the BB or AB haplotype is defined as the presence of one or more of the following genes: KIR2DL2, KIR2DL5, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS5, and KIR3DS1. The AA haplotype is defined by the absence of all the above mentioned genes and the presence of the following genes: KIR2DL1, KIR2DL3, KIR2DS4, and KIR3DL1. The KIR genotyping was performed using 11 homemade PCR reactions per individual, according to a previously described protocol (48). In each reaction, 2 µl of diluted DNA extract was amplified in 20 µl of SybrGreen-based PCR mix (IQ Sybr Green Supermix, BIORAD, CA, US) containing 0.3 pmol/µl of primers. The PCR was performed on a Light Cycler®96 Detection System (Roche Applied Science, Mannheim, Germany) in a 96 well-plate using a thermocycling profile as follows: 10 min at 95°C followed by 40 cycles of 95°C (15 seconds) and 62°C (60 seconds). Data were analyzed with Light Cycler®96 Software 1.1.

5.2.5. Statistical analysis

Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL, USA). The chi-square test was used to evaluate the observed and expected genotypes frequency, the number of HLA mismatches, missing-self (ms), non-inherited maternal antigens (NIMAs), and the combinations between maternal KIR with child HLA-C, in relation to symptoms at birth and LTI. With expected low values, a Fischer's exact test was used instead. The maternal and fetal HLA allele frequencies were tested for Hardy-Weinberg Equilibrium (HWE), stating that in the absence of other influences the genotype frequency in a certain population remains constant from generation to generation (49). In addition, a univariate logistic regression was performed to investigate potential predictors of symptoms at birth and LTI development. A p-value < 0.05 was considered statistically significant.

5.3. RESULTS

5.3.1. Study population and clinical data

The clinical data of the congenitally infected children included in this study are depicted in **Table 1**. A total of 96 mother-child pairs, were successfully typed for at least one of the HLA genes included in this study. Eight mother-child pairs could not be typed due to low DNA quality and concentration. Nineteen (20%) children had symptoms at birth, and 11 (58%) of those had LTI. In addition, 16 (21%) asymptomatic children had LTI. Overall, 27 (28%) of infected children developed any LTI.

5.3.2. Maternal and child HLA-C, HLA-E, and HLA-G genotypes in relation to cCMV clinical outcome

First, we evaluated whether the maternal genotype plays a role in cCMV outcome. For this purpose, we tested whether the genotype frequencies of the HLA-C groups, C1 and C2, and of the HLA-E and HLA-G alleles from the mothers were in Hardy-Weinberg equilibrium (HWE). The HLA-C groups of mothers with children symptomatic at birth were not in HWE (p=0.046) (Table S2 in Supplementary Material). In our cohort, a significantly higher percentage of mothers was homozygous for HLA-C1 in the group of children symptomatic at birth compared to the asymptomatic group, whereas the percentage of mothers homozygous or heterozygous for HLA-C2 was lower (Table 2).

Table 1. Characteristics and clinical outcome of study population

	Congenital CMV infection		
	Overall n = 96	**Asympt.**[1] n = 77	**Sympt.**[2] n = 19
Gender			
Male	57	44	13
Female	39	33	6
Gestational age (weeks)[3]	39 (28-42)	39 (37-42)	36 (28-41)
Birth weight (g)[3]	3435 (900-5110)	3540 (2635-5110)	2800 (900-4170)
Long term impairment			
Hearing impairment[4]	3	2	1
Visual impairment[5]	2	2	0
Neurological impairment[6]	5	2	3
Motor impairment[7]	13	9	4
Cognitive impairment[8]	7	4	3
Speech/language problem[9]	18	10	8
One or more impairment[10]	27	16	11

[1] Asymptomatic at birth; [2] Symptomatic at birth: premature (n=11), dysmature (n=2); microcephaly (n=5); neonatal hearing loss (n=1); abnormal cranial ultrasound (n=1); [3] Values are medians with minimum and maximum; [4] sensorineural hearing loss; [5] optic nerve atrophy (n=1), cortical visual impairment (n=1); [6] cerebral palsy (n=1), epilepsy (n=1), microcephaly (n=1), ADHD (n=1), autism (n=3); [7] motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder; [8] cognitive impairment based on test or diagnosis; [9] language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder; [10] Any long-term impairment, in one or more domains.

The functional difference between HLA-C1 and HLA-C2 suggests a possible association of these alleles with clinical outcome in our cohort (23, 50, 51), with absence of maternal HLA-C2 being a risk factor. Indeed, the odds of having symptomatic neonates was higher when mothers were homozygous for HLA-C1 than when mothers were homozygous for HLA-C2 or heterozygous (OR = 3.950, 95% CI 1.378 – 11.320, p=0.011). Consequently, only 5.3% of children symptomatic at birth were homozygous for HLA-C2 (**Table 2**).

The genotype frequencies of HLA-E and HLA-G alleles from the mothers were in HWE in all groups (Table S2 in Supplementary Material). Despite this, a significantly higher percentage of mothers homozygous for HLA-G deletion (HLA-G del/del) was observed in the group of children that developed LTI compared to those who did not; this percentage was also higher in the symptomatic group compared to the asymptomatic group, though not significant (**Table 3**). HLA-G del/del is

Table 2. HLA-C, KIR and cCMV clinical outcome

	Symptoms at birth			Long-term impairments (LTI)		
	Symptomatic % n = 19	Asymptomatic % n = 76	p-value Chi	LTI (≥ 1)[1] % n = 26	No LTI[2] % n = 69	p-value Chi
HLA-C Genotype						
Mother			**0.021***			0.084
C1C2	21.1	52.6		38.5	49.3	
C1C1	63.2	30.3		30.8	39.1	
C2C2	15.8	17.1		30.8	11.6	
Child			0.371			0.712
C1C2	52.6	46.1		50	46.4	
C1C1	42.1	35.5		30.8	39.1	
C2C2	5.3	18.4		19.2	14.5	
HLA-C mm[3]	84.2	68.4	0.172	80.8	68.1	0.223
HLA-C1 mm[4]	15.8	11.8	0.701˜	19.2	10.1	0.300˜
HLA-C2 mm[5]	31.6	10.5	**0.031˜***	19.2	13	0.519˜
HLA-C ms[6]	78.9	72.4	0.560	96.2	65.2	**0.002***
HLA-C1 ms[7]	5.3	13.2	0.454˜	7.7	13	0.722˜
HLA-C2 ms[8]	10.5	18.4	0.514˜	19.2	15.9	0.761˜
KIR haplotype mother[9]			0.659			0.356
A	36.8	31.5		40	29.9	
B	63.2	68.5		60	70.1	
Maternal KIR A– Child C2[10]	21.1	20.5	1.000˜	20	20.9	0.925

[1] Any long-term impairment, in one or more domains of impairments: hearing, visual neurologic, motor, cognitive, and speech-language; [2] Absence of any long-term impairment; [3] HLA-C mm: HLA-C mismatches; [4] HLA-C1 mm: HLA-C mismatches in the C1 group, the mother is homozygous for C2 and the child is heterozygous; [5] HLA-C2 mm: HLA-C mismatches in the C2 group, the mother is homozygous for C1 and the child is heterozygous; [6] HLA-C ms: HLA-C missing-self; [7] HLA-C1 ms: HLA-C missing-self in the C1 group, the mother is heterozygous and the child is homozygous for HLA-C2; [8] HLA-C2 ms: HLA-C missing-self in the C2 group, the mother is heterozygous and the child is homozygous for HLA-C1; [9] Maternal KIR A haplotype is defined as the absence of activating KIRs (AA) and KIR B haplotype as the presence of different numbers of activating KIRs (AB or BB); [10] Combination of maternal KIR A haplotype with HLA-C2 positive child (either C1C2 or C2C2); [9,10] N asymptomatic = 73, N with LTI = 25, N without LTI = 67. ˜ Fischer's exact test used. * p < 0.05.

Table 3. HLA-G and cCMV clinical outcome

	Symptoms at birth			Long-term impairments (LTI)		
	Symptomatic % n = 19	Asymptomatic % n = 77	p-value Chi	LTI (≥ 1)[1] % n = 27	No LTI[2] % n = 69	p-value Chi
HLA-G Genotype						
Mother			0.174			**0.023***
del/del	52.6	29.9		55.6	26.1	
ins/ins	10.5	15.6		11.1	15.9	
del/ins	36.8	54.5		33.3	58	
Child			0.459			0.514
del/del	26.3	32.5		37	29	
ins/ins	10.5	19.5		11.1	20.3	
del/ins	63.2	48.1		51.9	50.7	
HLA-G del mm[3]	5.3	10.4	0.683˜	3.7	11.6	0.437˜
HLA-G ins mm[4]	31.6	11.7	0.070˜	25.9	11.6	0.116˜
HLA-G del ms[5]	5.3	14.3	0.449˜	3.7	15.9	0.169˜
HLA-G ins ms[6]	5.3	14.3	0.449˜	7.4	14.5	0.500˜

[1] Any long-term impairment, in one or more domains of impairments: hearing, visual neurologic, motor, cognitive, and speech-language; [2] Absence of any long-term impairment; [3] HLA-G del mm: HLA-G deletion mismatches, the mother is homozygous for HLA-G insertion and the child is heterozygous; [4] HLA-G ins mm: HLA-G insertion mismatches, the mother is homozygous for HLA-G deletion and the child is heterozygous; [5] HLA-G del ms: HLA-G deletion missing-self, the mother is heterozygous and the child is homozygous for HLA-G insertion; [6] HLA-G ins ms: HLA-G insertion missing-self, the mother is heterozygous and the child is homozygous for HLA-G deletion. ˜ Fischer's exact test used. * $p < 0.05$.

related to higher HLA-G protein levels (52, 53), soluble and possibly membrane-bound; therefore our findings suggest that the functional difference of the two alleles is associated with clinical outcome, with the maternal homozygous status being a risk factor. Indeed, the odds of developing LTI was higher when mothers were HLA-G del/del (OR 3.542, 95% CI 1.397 – 8.977, p=0.008). Likewise, the odds of having symptoms at birth were higher when mothers were HLA-G del/del, although not significant (not shown). Next, given the role of HLA-G during CMV infection, the HLA-G del/del polymorphism was assessed in relation to CMV viral load. For this purpose, the study group was divided into two groups according to the viral load measured in DBS namely low (< 500 IU/ml) and high (> 500 IU/ml) viral load groups. A higher percentage of mothers with HLA-G del/del was observed in the high viral load group compared to the low viral load group (41.4 and 12.0% respectively, p=0.008), whereas this was not observed in the children (30.0 and 32.0% respectively, p=0.852) (Table S3 in Supplementary Material).

Finally, we evaluated whether the child's genotype plays a role in cCMV outcome. The genotype frequencies of the HLA-C groups, and HLA-G alleles in the children were in HWE in all groups, whereas the HLA-E alleles were not, both in the symptomatic and asymptomatic groups (p=0.037 and p=0.024, respectively) (Table S2 in Supplementary Material). A higher percentage of children heterozygous for HLA-E was observed in the symptomatic group compared to the asymptomatic group whereas the percentage of homozygotes, either HLA-E*0101 or HLA-E*0103, was lower

(**Table 4**). HLA-E*0103 is associated with higher proteins levels (40); therefore our findings suggest that the functional difference of the two alleles is not associated with clinical outcome.

5.3.3. Maternal-fetal HLA-C, HLA-E, and HLA-G mismatches in relation to cCMV clinical outcome

We next investigated whether maternal-fetal HLA mm are associated with a worse cCMV outcome. For this purpose, mm were calculated on the basis of the presence of an Ag in the fetus, which was absent in the mother because the inherited paternal antigen (IPA) was different from both maternal alleles (**Table 5**). The mm were compared between children symptomatic and asymptomatic at birth, as well as between children who developed LTI and those who did not. A significantly higher percentage of HLA-C2 mm was observed in the symptomatic group compared to the asymptomatic group (**Table 2**). Most likely, the mm derived from the higher percentage of mothers homozygous for HLA-C1 while a higher percentage of symptomatic neonates was heterozygous (**Table 2**). Therefore, the functional consequence of HLA-C2 absence in the mother might be the main risk factor, even though the odds of having symptoms at birth were significantly higher as well with HLA-C2 mm (OR = 3.923, 95% CI 1.166-13.201, p=0.027).

To exclude the possibility that symptoms at birth were associated with specific HLA-C KIR combinations rather than maternal genotypes or maternal-fetal mm, the number of HLA-C1/HLA-C2 KIR epitope combinations were analyzed. No differences in preferential fetal HLA-C/maternal KIR combinations were observed (**Table 2**). In addition, a significantly higher percentage of HLA-E*0101 mm was observed in the symptomatic group compared to the asymptomatic (**Table 4**). The HLA-E mm may be driven by the neonatal aberrant distribution of the genotypes. However, because the difference in HLA-E genotypes would probably have no functional consequences, the mm may be the primary risk factor. Indeed, the odds of developing symptoms at birth were significantly higher when HLA-E*0101 mm were present (OR = 4.343, 95% CI 1.357- 13.897, p=0.013). In addition, the HLA-E*0101 mm was assessed in relation to CMV viral load, and a higher percentage of HLA-E*0101 mm was found in the high viral load group compared to the low viral load group (23.2% and 0.0% respectively, p=0.005) (Table S3 in Supplementary Material). Finally, no differences in HLA-G mm were observed with respect to symptoms nor to LTI development (**Table 3**).

5.3.4. HLA-C, HLA-E and HLA-G missing-self/NIMAs in relation to cCMV clinical outcome

We finally assessed whether missing-self/NIMAs influence cCMV clinical outcome. For this purpose, missing-self was defined as an Ag in the mother, which was absent in the child because the IPA was different from the NIMA (**Table 5**). From the mothers' perspective, missing-self implies a mechanism of recognition by dNK of trophoblasts lacking maternal self-molecules. Missing-self does not necessarily indicate mismatches because the IPA could still be the same as the inherited maternal antigen (**Table 5**). The maternal Ag, that was missing in the child, is considered by the child a NIMA. Therefore, despite being the same aforementioned Ag, other mechanisms than recognition by dNK of missing-self are in place. Hence, we will refer to this Ag, triggering different responses, as missing-

Table 4. HLA-E and cCMV clinical outcome

	Symptoms at birth			Long-term impairments (LTI)		
	Symptomatic % n = 19	Asymptomatic % n = 76	p-value Chi	LTI (≥ 1)[1] % n = 26	No LTI[2] % n = 69	p-value Chi
HLA-E Genotype						
Mother			0.499			0.809
0101/0101	21.1	27.6		23.1	27.5	
0103/0103	36.8	23.7		30.8	24.6	
0101/0103	42.1	48.7		46.2	47.8	
Child[7]			**0.015***			0.714
0101/0101	15.8	35.5		33.3	30.9	
0103/0103	10.5	27.6		18.5	26.5	
0101/0103	73.7	36.8		48.1	42.6	
HLA-E*0101 mm[3]	36.8	11.8	**0.016~***	26.9	13	0.129~
HLA-E*0103 mm[4]	15.8	9.2	0.413~	15.4	8.7	0.453~
HLA-E*0101 ms[5]	10.5	17.1	0.728~	15.4	15.9	1.000~
HLA-E*0103 ms[6]	10.5	17.1	0.728~	23.1	13	0.343~

1 Any long-term impairment, in one or more domains of impairments: hearing, visual neurologic, motor, cognitive, and speech-language; 2 Absence of any long-term impairments; 3 HLA-E*0101 mm: HLA-E*0101 mismatches, the mother is homozygous for HLA-E*0103 and the child is heterozygous; 4 HLA-E*0103 mm: HLA-E*0103 mismatches, the mother homozygous for HLA-E*0101 and the child is heterozygous; 5 HLA-E*0101 ms: HLA-E*0101 missing-self, the mother is heterozygous and the child is homozygous for HLA-E*0103; 6 HLA-E*0103 ms: HLA-E*0103 missing-self, the mother is heterozygous and the child is homozygous for HLA-E*0101. 7 N=27 neonates with LTI, and N=68 neonates without LTI. ~ Fischer's exact test used. * $p < 0.05$.

Table 5. Definitions: mismatches, missing-self and NIMAs.

Genotype combinations[1]		Maternal perspective		Child perspective
Maternal genotype	**Fetal genotype**	**Mismatch (mm)[2]**	**Missing-self (ms)[3]**	**NIMAs[4]**
a/b	a/a	no	yes	yes
a/b	b/b	no	yes	yes
a/b	a/c	yes	yes	yes
a/b	c/b	yes	yes	yes
a/a	a/b	yes	no	no
b/b	a/b	yes	no	no
a/a	a/a	no	no	no
a/b	a/b	no	no	no
b/b	b/b	no	no	no

[1] Combinations of maternal and child HLA genotype by using 3 hypothetical genes (a, b, c); [2] Mismatches (mm) are defined as the Ag that the child has but the mother does not have, because the inherited paternal antigen (IPA) is different from both maternal alleles; [3] Missing-self (ms) is defined as the Ag that the mother has and the child does not because the inherited paternal antigen (IPA) differs from the non-inherited maternal antigen (NIMAs); [4] Non-inherited maternal antigens (NIMAs) are defined as the Ag that the child did not inherit but is exposed to, due to maternal microchimerism.

self/NIMAs. In our cohort, there were no significant differences in HLA-C, HLA-E, and HLA-G missing-self/NIMAs in relation to symptoms at birth. However, for LTI development, a significantly higher percentage of HLA-C missing-self/NIMAs, but not of HLA-E and HLA-G missing-self/NIMAs, was observed (**Tables 2-4**). The aforementioned results suggest that the HLA-C missing-self/NIMA may be considered as a risk factor for LTI development, rather than for symptoms at birth. Indeed, the odds of developing LTI was significantly higher when the child had HLA-C mismatched NIMAs (OR 13.3, 95% CI 1.701 – 104.535, p=0.014).

5.4. DISCUSSION

To gain more insights into cCMV pathogenesis and its clinical consequences, the role of HLA-C, HLA-E, and HLA-G genotypes was evaluated in a large cohort of children with cCMV and their mothers. HLA-C, HLA-E, and HLA-G are the only HLA molecules expressed by the trophoblasts, and therefore they might play a role in cCMV outcome. To systematically discuss the findings, they will be described in relation to the compartments involved in the virus-host interaction, which are maternal, placental, fetal, and child.

First of all, the nature of maternal infection and her immune response can influence cCMV outcome. In our cohort, the maternal HLA-G del/del genotype was associated with a worse cCMV outcome. This could be explained by the immunosuppressive effect of this genotype which is associated with the presence of higher HLA-G proteins levels, soluble and possibly membrane-bound (52, 53). Indeed, HLA-G can inhibit various immune cell subsets (16, 17). The hypothetical model to account for maternal HLA-G genotype in relation to cCMV outcome is depicted in **Figure 1**. In addition, CMV induces up-regulation of HLA-G in order to escape the host defense and, after an initial local replication, CMV dissemination is likely to be cell-associated, occurring mainly through endothelial and monocytes/macrophages (54). Hence, higher HLA-G protein levels might create a favorable environment for CMV, facilitating replication and dissemination. Interestingly, this HLA-G del/del polymorphism has been associated to active CMV infection with higher viral loads in children (29). In our cohort, maternal samples during pregnancy were not available. However, neonatal CMV viral load determined in DBS was related to the HLA-G genotype of the mother, as reflected by the higher percentage of mothers HLA-G del/del in the high viral load group compared to the low viral load group (Table S3 in Supplementary Material). This suggests that a reduced maternal control of CMV infection may increase the viral loads at the placenta and, consequently, in the fetus. Placental CMV infection triggers an inflammatory response that alters the trophoblast, inducing placental dysfunction and fetal impairments, such as intrauterine growth retardation (55-57). Interestingly, in a group of pregnant women with primary CMV infection and neonates with CMV disease, the placental thickness was increased (58). In addition, in another cohort of pregnant women with primary CMV infection, increased sHLA-G levels were observed in both maternal serum and amniotic fluid of symptomatic fetuses (30). However, no information was available on the maternal and child HLA-G genotypes.

Vertical transmission occurs through placental infection, which therefore was considered after the maternal immune response. The hypothetical model to account for our findings in the placenta in relation to cCMV outcome is shown in **Figure 2**. First of all, a significantly higher percentage of

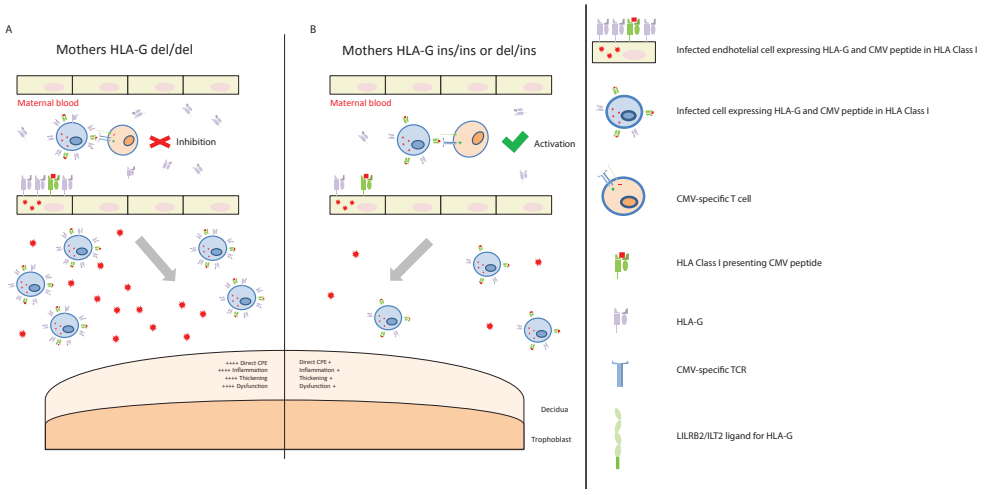

Figure 1. Placental CMV infection according to maternal HLA-G genotype. **A.** Mothers homozygous for HLA-G deletion are predisposed to having higher levels of sHLA-G, and possibly membrane-bound HLA-G, proteins in those tissues where HLA-G is expressed, such as endothelial cells and monocytes/macrophages. When maternal T cells encounter an infected cell with higher levels of HLA-G, it is inhibited. This creates a favorable environment for CMV replication and dissemination. Consequently, more extensive infection of the placenta leads to increased CPE, inflammation, thickening and dysfunction. This in turn contributes to a worse outcome. **B.** Mothers homozygous for HLA-G insertion, or heterozygous, are predisposed to having lower levels of sHLA-G, and possibly membrane-bound HLA-G, proteins. When maternal T cells encounter an infected cell with lower levels of HLA-G protein, it is activated. CMV replication and dissemination is therefore contained. Consequently, lower numbers of infected cells and viral loads reach the placenta, with a reduced CPE, inflammation, thickening and dysfunction. This in turn contributes to a better outcome.

symptomatic neonates had HLA-E*0101 mm, and a higher extent of HLA-C mm. HLA mm have been shown to result in the occurrence of maternal fetus-specific T cells, both in the maternal peripheral blood and at the maternal-fetal interface (20, 59). In normal conditions, this does not impair pregnancy because there is a parallel increase of regulatory mechanisms modulating such responses (20). However, viral infections can increase the levels of pro-inflammatory cytokines, chemokines, and the influx of T cells in decidual tissues (60, 61). In this situation, the regulatory mechanisms might not be able to efficiently inhibit allogeneic lymphocytes (19), which could damage the placenta. Furthermore, because CMV peptides can be presented in the context of HLA-C and HLA-E (62-64), mm could lead to suboptimal viral clearance at the placenta, as maternal CMV-specific cells would not efficiently recognize CMV presented in the context of allo-HLA. This has been described in allogeneic hematopoietic stem cell transplantation, where the clinical activity of donor-derived virus-specific T cells can be abolished if the immunodominant T cells are restricted by HLA not shared by the host (65). Consequently, this may result in higher placental and fetal viral loads. Indeed, a higher percentage of maternal-fetal HLA-E*0101 mm was found in the high viral load group compared to the low viral load group, and the same was observed for HLA-C mm (76.8% and 56.0% respectively, p=0.049) (Table S3 in Supplementary Material). Finally, dNK cells are the predominant

leukocyte population at the placenta, and they play a central role in the immune cross-talk and in the placentation process. The combination of maternal AA KIR with fetal HLA-C2 was associated with increased risk of pre-eclampsia, as it led to the absence of activated dNK and poor placentation (23). Indeed, HLA-C2 has a stronger inhibition capacity when binding to its inhibitory KIR (KIR2DL1) than C1 with its corresponding receptor (KIR2DL2/3) (23, 50, 51). In our cohort, clinical outcome was not associated with specific maternal KIR-fetal HLA-C combinations. Rather, the absence of maternal HLA-C belonging to the C2 group was associated with a worse cCMV outcome at birth. In the absence of HLA-C2, the activating signals on dNK cells may prevail the inhibitory signals, which in turn promote a pro-inflammatory response. In addition, it has been shown that expression of KIR2DS1 by dNK increases their cytotoxic function towards infected maternal decidual stromal cells, which could possibly lead to a reduction of placental virus-induced pathology (24). In our cohort, the presence of the maternal gene KIR2DS1 was not associated with a better short- and long-term outcome (Table S4 in Supplementary Material). However, these results would need to be confirmed as the lack of statistical power may have been a limiting factor in detecting a small effect. Taken together, the aforementioned mechanisms may contribute to the complex multifactorial process of placental immunopathology and dysfunction, which has a direct impact on outcome at birth. Importantly, besides the fact that placental inflammation has been described in relation to cCMV (55-57), prematurity has also been associated to chronic placental inflammation in the absence of infections (66). Given the relatively high percentage of premature infants with cCMV in our cohort, we evaluated whether the aforementioned markers shown to be associated with symptoms may have been influenced by this. After excluding the 11 premature infants, a slight change in *p*-values, but with the same trend of percentages, was observed in relation to symptoms at birth (data not shown), most likely due to the lack of statistical power. This suggests that we cannot fully exclude that the relatively high percentage of premature infants in the symptomatic group partially influenced the association between the aforementioned biomarkers and symptoms. Taking into account the association between cCMV and prematurity (67), it may be plausible to assume that prematurity is an effect of both cCMV and the aforementioned HLAs.

The child's immune response was considered after the placental compartment, and the hypothetical model to account for our findings in relation to cCMV outcome is shown in **Figure 3**. Although certain cCMV clinical consequences are present directly after birth, some of the permanent impairments have an onset in the first years after birth, or may progress during childhood (1). The late-onset hearing loss is commonly considered as the result of a chronic disease syndrome, of which the molecular mechanisms have not been elucidated yet, though data suggest they are due to a chronic productive infection throughout childhood (7, 8). For this reason, the child's immune response to CMV may play a central role in preventing LTI. In our cohort, a high percentage of children with LTI had maternal-fetal HLA-C NIMAs. Their role in LTI development may lie in the tolerance induced in the fetus towards NIMAs. Indeed, during pregnancy and after birth, the child is exposed to maternal allogeneic cells because of the transfer of maternal cells to the fetus, namely maternal microchimerism (68, 69). The developing neonatal immune system does not consider NIMAs as non-self, but rather develops long-lasting regulatory mechanisms to prevent an immune response against NIMAs. The NIMA effect has mainly been shown in transplantation.

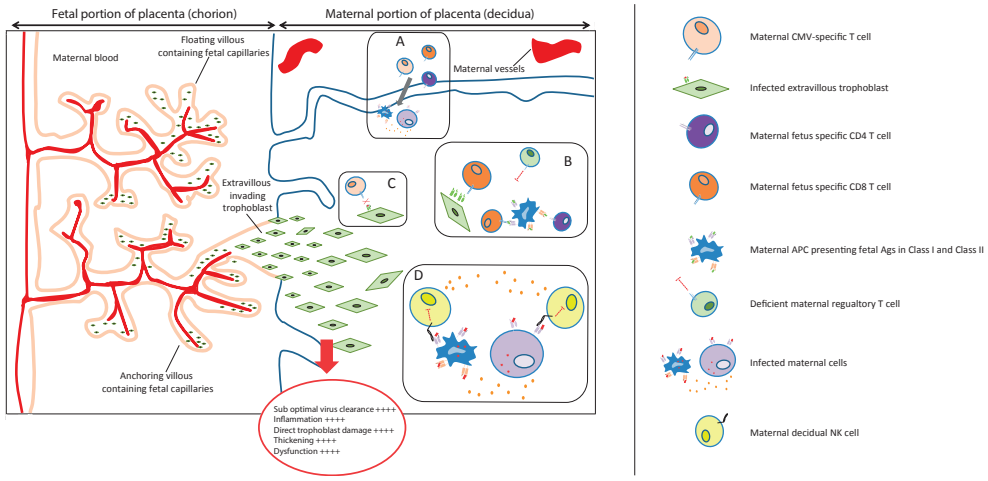

Figure 2. Placental immunopathology. **A.** Viral infections can increase the levels of pro-inflammatory cytokines and chemokines, and increase the influx of T cells in decidual tissues (60, 61). **B.** The regulatory mechanisms might not be able to efficiently modulate the maternal fetus-specific lymphocytes, specific for HLA-C and HLA-E, which can therefore directly or indirectly recognize trophoblast cells. **C.** HLA-C and HLA-E mm could prevent an optimal viral clearance because maternal CMV-specific cells would not efficiently recognize CMV presented in the context of allo-HLA. **D.** In the absence of maternal HLA-C2, the balance between activating and inhibitory signals on dNK cells may be more skewed towards dNK cells activation, because HLA-C1 has less inhibitory capacity on dNK. This activation could in turn promote a pro-inflammatory response, exacerbating placental immunopathology.

The survival rate of kidney grafts with a mismatched Ag identical to the recipient's NIMA was better than in situations where the mismatched Ag was not a NIMA (70, 71). Similar effects have been shown in case of stem cell, cord blood and bone marrow transplantation (72-74). Fetal T cells can recognize NIMAs presented by maternal cells *via* the shared HLA, or by fetal APC in the context of HLA class I and class II. One of the first steps in the tolerance towards NIMAs is the induction of NIMA-specific Tregs, which suppress the fetal antimaternal immunoresponse and persist at least till early adulthood (75, 76). In addition, tolerogenic APCs presenting NIMAs are induced, and suppress NIMAs-specific T cells (77, 78). These mechanisms may influence the child's immune response. First, the NIMA-specific Tregs may create a general tolerogenic environment that indirectly impair the immune response to CMV. Second, through a mechanism called linked immunosuppression, a certain level of tolerance specific for CMV can be induced (78). Indeed, fetal APCs could present both NIMAs and CMV Ag, as maternal cells can be infected or because maternal cells reside in infected fetal tissues. This may result in a less efficient antiviral response during childhood, which possibly leads to a more persistent viral replication and tissue damage. Therefore, while the NIMA effect may be beneficial in transplantation, it could prevent an optimal antiviral response due to a partial tolerance to CMV as well. Similar to symptoms at birth, we evaluated whether the relatively high percentage of premature infants in the symptomatic group may have influenced the results relative to LTI. After excluding the premature infants, the results and significance did not change

(data not shown), suggesting that the relatively high percentage of premature does not influence the association between HLA-C NIMAs and LTI development.

This study is not without limitations. First of all, potential effects of CMV on the expression of the studied HLA Ags could not be taken into account. Indeed, CMV has developed strategies to evade host immunity and to establish latency, e.g. by down-regulating classical HLA molecules and up-regulating non-classical HLA (54, 79-81). Second, a CMV-independent role of these HLA combinations in adverse pregnancy outcome cannot be totally excluded. However, we did not observe the previously described HLA KIR combinations associated with pregnancy complications, further suggesting that our observations specifically apply to cCMV.

To the best of our knowledge, this is the first study on maternal-fetal HLA in a large cohort of children with cCMV. If our hypotheses are correct, symptoms at birth are mainly caused by the immunopathology that takes place at the maternal-fetal interface, as a result of a multifactorial process of sub-optimal viral clearance, enhanced allo-reactivity, and increased inflammation. Whereas the inefficient long-term control of CMV infection, that plays a role in LTI development, might have been partially caused by the NIMA effect. This study gives useful insights and generates new hypotheses on cCMV pathogenesis in all compartments involved during cCMV. Furthermore, if confirmed in other cohorts, these findings could be evaluated as potential prognostic markers for clinical outcome. Indeed, a reliable marker for cCMV outcome could provide the means to introduce the long-debated newborn screening program for CMV in DBS by defining subgroups of neonates that would benefit from clinical, audiological follow-up, and possibly antiviral treatment (82).

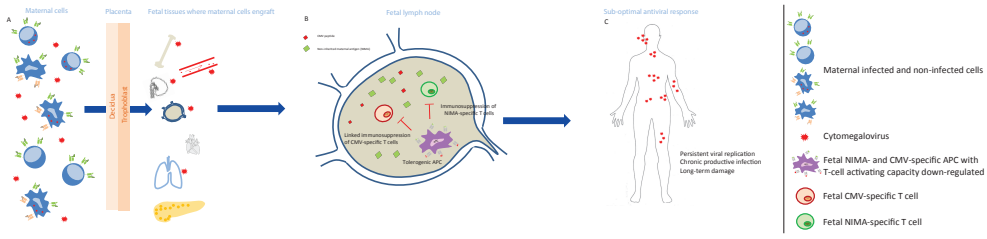

Figure 3. NIMA effect and linked immunosuppression in cCMV long-term outcome. **A.** During pregnancy, maternal cells pass through the placenta to the fetus and can engraft in several fetal tissues, that may be infected by CMV, and persist at least till adulthood. Maternal cells can be either CMV infected or not, and can carry HLA Ags that the child does not have, i.e. NIMAs. **B.** After NIMAs recognition by fetal T cells, tolerogenic APCs, with reduced T cell activating capacity, are induced in order to suppress a fetal anti-maternal immune response. These tolerogenic APCs may be also specific for CMV, as maternal cells can be infected or can reside in infected fetal tissues, and suppress CMV-specific T cells (linked immunosuppression). **C.** The antiviral response in the child is therefore less efficient, possibly leading to a more persistent viral replication with a chronic productive infection, and tissue damage.

5.5. CONFLICT OF INTEREST

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

5.6. AUTHOR CONTRIBUTIONS

RR and AV designed research; RR, DR and ME performed and supervised the experiments; RR and GH analyzed the data; RR and FC interpreted the results in relation to the possible mechanisms; RR and AV wrote the manuscript; FC, GH, AK and ME revised the manuscript critically.

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5.9. ETHICS STATEMENT

This study was approved by the Medical Ethics Committee of the Leiden University Medical Center, and all the parents of the children included have given written informed consent in accordance with the Declaration of Helsinki.

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SUPPORTING DATA

Table S1. HLA-C frequency in 5604 randomly selected healthy Dutch blood donors

HLA-C alleles	Positive	Negative	%
C*01	323	5281	5,8%
C*02	575	5029	10,3%
C*03	1665	3939	29,7%
C*04	1287	4317	23,0%
C*05	764	4840	13,6%
C*06	898	4706	16,0%
C*07	3140	2464	56,0%
C*08	233	5371	4,2%
C*12	466	5138	8,3%
C*14	117	5487	2,1%
C*15	287	5317	5,1%
C*16	343	5261	6,1%
C*17	97	5507	1,7%
C*18	2	5602	0,0%

Table S2. Hardy-Weinberg Equilibrium and cCMV clinical outcome

	Symptoms at birth						Long-term impairments (LTI)					
	Sympt.			Asympt.			LTI (≥ 1)			No LTI		
	Obs.[1]	Exp.[2]	p-value[3]	Obs.	Exp.	p-value	Obs.	Exp.	p-value	Obs.	Exp.	p-value
HLA-C Genotype												
Mother			0.046			0.535			0.239			0.581
C1C2	4	7.4		40	37.3		10	13		34	31.9	
C1C1	12	10.3		23	24.3		8	6.5		27	28.1	
C2C2	3	1.3		13	14.3		8	6.5		8	9.1	
Child			0.342			0.655			0.945			0.917
C1C2	10	8.2		35	36.9		13	12.8		32	32.4	
C1C1	8	8.9		27	26.1		8	8.1		27	26.8	
C2C2	1	1.9		14	13.1		5	5.1		10	9.8	
HLA-E Genotype												
Mother			0.552			0.828			0.716			0.723
0101/0101	4	3.4		21	20.5		6	5.5		19	18.3	
0103/0103	7	6.4		18	17.5		8	7.5		17	16.3	
0101/0103	8	9.3		37	37.9		12	12.9		33	34.5	
Child			0.037			0.024			0.936			0.230
0101/0101	3	5.3		27	22.1		9	8.9		21	18.5	
0103/0103	2	4.3		21	16.1		5	4.9		18	15.5	
0101/0103	14	9.5		28	37.8		13	13.2		29	33.9	
HLA-G Genotype												
Mother			0.649			0.318			0.379			0.154
del/del	10	9.6		23	25.1		15	14.1		18	20.9	
ins/ins	2	1.6		12	14.1		3	2.1		11	13.9	
del/ins	7	7.8		42	37.7		9	10.8		40	34.1	
Child			0.198			0.844			0.561			0.853
del/del	5	6.4		25	24.6		10	10.7		20	20.4	
ins/ins	2	3.4		15	14.6		3	3.7		14	14.4	
del/ins	12	9.3		37	37.8		14	12.6		35	34.2	

[1] Obs: observed number of subjects; [2] Exp: expected number of subjects; [3] Chi-square test.

Table S3. HLA-G deletion genotype, HLA-E and HLA-C mm in relation to viral load

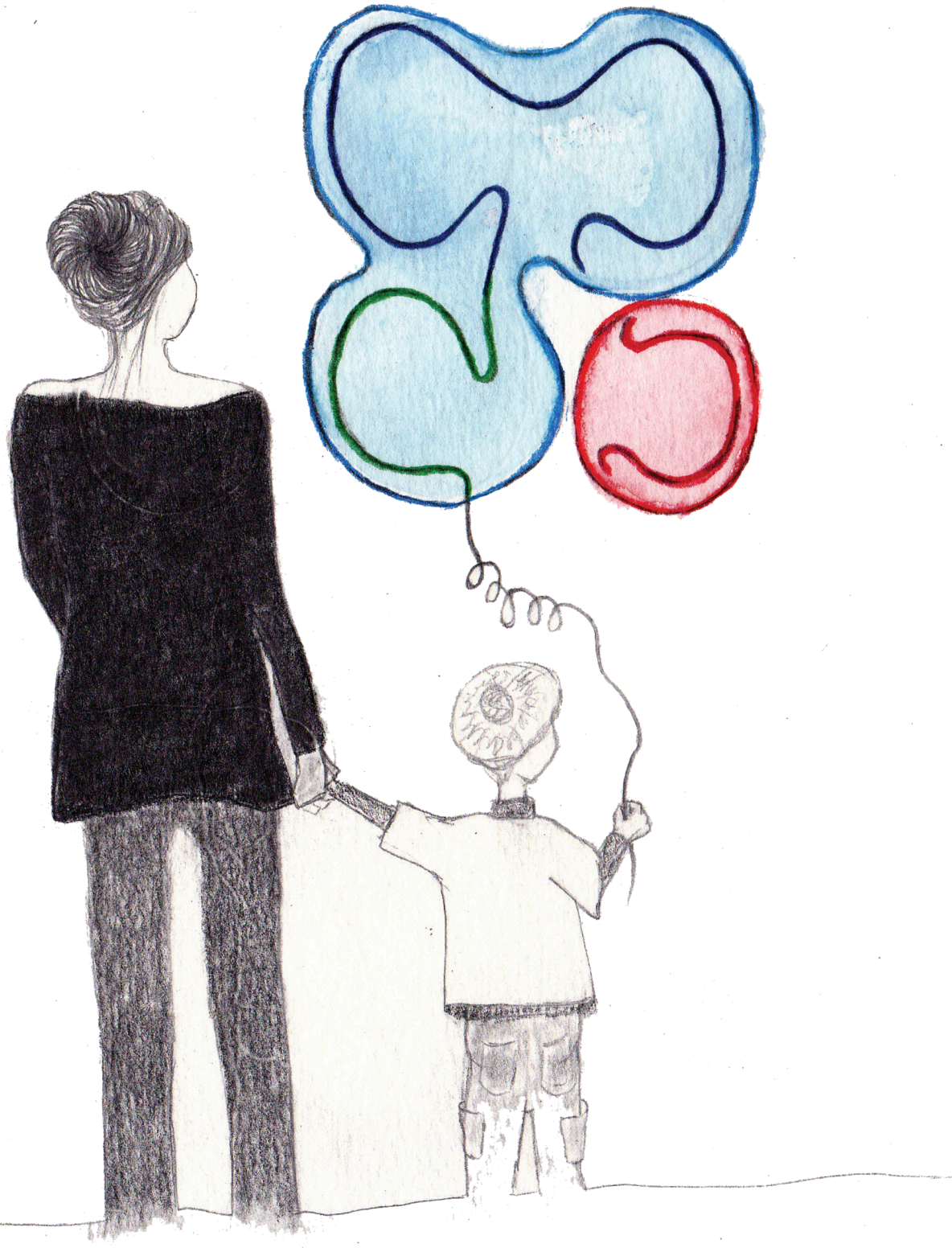
	Viral load[1]		
	Low[2] % n = 25	High[3] % n = 70	p-value Chi
HLA-G del/del mother[4]	12.0	41.4	**0.008***
HLA-G del/del child[5]	32.0	30.0	0.852
HLA-E*0101 mm[6]	0.00	23.2	**0.005~***
HLA-C mm[7]	56.0	76.8	**0.049***

[1] The study group was divided in two groups according to the CMV viral load measured in DBS. For one child the DBS was not available and the viral load could not be assessed; [2] Low: viral loads in DBS < 500 IU/ml; [3] High: viral loads in DBS ≥ 500 IU/ml; [4] Mothers homozygous for HLA-G deletion; [5] Children homozygous for HLA-G deletion; [6] HLA-E*0101 mm: HLA-E*0101 mismatches, the mother is homozygous for HLA-E*0103 and the child is heterozygous; [7] HLA-C mm: HLA-C mismatches; [6,7] N high group = 69. ~ Fischer's exact test used. * p < 0.05.

Table S4. Individual KIRs and cCMV clinical outcome

	Symptoms at birth			Long-term impairments (LTI)		
KIRs	Symptomatic % n = 19	Asymptomatic % n = 73	p-value Chi	LTI (≥ 1)[1] % n = 25	No LTI[2] % n = 67	p-value Chi
KIR2DL1	100.0	89.0	0.198~	100.0	88.1	0.102~
KIR2DL2	47.4	53.4	0.638	44.0	55.2	0.338
KIR2DL3	100.0	86.3	0.115~	92.0	88.1	0.723~
KIR2DL5	42.1	47.9	0.649	52.0	44.8	0.537
KIR2DS1	42.1	34.2	0.525	40.0	34.3	0.614
KIR2DS2	47.4	54.8	0.563	44.0	56.7	0.277
KIR2DS3	21.1	21.9	1.000~	32.0	17.9	0.145
KIR2DS4	100.0	97.3	1.000~	100.0	97.0	1.000~
KIR2DS5	36.8	30.1	0.575	40.0	28.4	0.285
KIR3DL1	100.0	97.3	1.000~	100.0	97.0	1.000~
KIR3DS1	42.1	35.6	0.602	44.0	34.3	0.393

[1] Any long-term impairment, in one or more domains of impairments: hearing, visual neurologic, motor, cognitive, and speech-language; [2] Absence of any long-term impairment; ~ Fischer's exact test used.



CHAPTER 6

MATERNAL AND CHILD HUMAN LEUKOCYTE ANTIGENS IN CONGENITAL CYTOMEGALOVIRUS INFECTION

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ABSTRACT

Congenital Cytomegalovirus infection (cCMV) is the most common cause of congenital infections worldwide causing permanent long-term impairment (LTI). cCMV immunopathogenesis remains largely unknown due to the complex interplay between viral, maternal, placental and child factors. The aim of this study was to determine the possible role of particular HLA antigens, of the number of HLA mismatches (mm) and non-inherited maternal antigens (NIMAs) in a large retrospective nation-wide cohort of children with cCMV and their mothers. HLA Class I (HLA-A, HLA-B and HLA-C) and HLA Class II (HLA-DR and HLA-DQ) were assessed in 96 mother-child pairs in relation to a control group of 5604 Dutch blood donors, but no significant differences were observed. Next, although these HLA antigens could not be assessed in relation to symptoms at birth, nor to LTI, due to the low number of cases, they could be evaluated in relation to CMV viral load. HLA-DRB1*04, and potentially HLA-B*51, was shown to have a protective role in the children as its frequency was increased in the low viral load group compared to the high viral load group, and this remained significant after correction. The number of HLA mm and of NIMAs were not associated to symptoms at birth nor to LTI or viral load. In conclusion, although none of the HLA alleles could be put forward as prognostic marker for long-term outcome, our findings give useful insights into cCMV pathogenesis, and identify potential HLAs that correlate with a better viral control.

6.1. INTRODUCTION

Congenital cytomegalovirus infection (cCMV) is the most common congenital infection in industrialized countries with an overall birth prevalence between 0.6 and 0.7% (Dollard et al., 2007, Kenneson and Cannon, 2007). Despite the considerable knowledge of cCMV clinical outcome, the multifactorial process that determines whether a neonate will have symptoms at birth or will develop permanent long-term impairment (LTI) is still poorly understood. In 12.7% of neonates, cCMV leads to symptoms at birth, such as petechiae, jaundice, hepatosplenomegaly, and microcephaly (Kenneson and Cannon, 2007, Dollard et al., 2007). An estimated 40-58% of these symptomatic neonates will develop LTI, such as hearing loss, cognitive and motor delay (Dollard et al., 2007). Importantly, of the 87.3% asymptomatic neonates at birth, around 13.5% will develop permanent sequelae (Dollard et al., 2007).

cCMV pathogenesis is the result of a complex multifactorial process that comprises maternal, placental, fetal and child factors. The nature of maternal infection and her immune response have an important role in cCMV and its outcome. Indeed, the risk of vertical transmission is 30-40% among women without prior CMV infection, while among previously exposed women this risk is at least 10-fold lower (Kenneson and Cannon, 2007). The risk of symptomatic CMV disease is mainly associated to maternal infection occurring in the first, and second trimester of pregnancy (Pass et al., 2006, Enders et al., 2011). Although previous studies have demonstrated a fetal and neonatal CMV-specific immune response, its role in controlling CMV disease still needs to be clarified (Vermijlen et al., 2010, Lidehall et al., 2013, Hassan et al., 2007).

The CMV-specific T cell mediated immune response is dependent of the HLA type of the individual (Goldrath and Bevan, 1999, Davis and Bjorkman, 1988). CMV-specific T cells restricted to certain HLA alleles may mount a more effective immune response than others. In transplantation, several HLA alleles have been shown to have a central role in CMV infection and disease (Du et al., 2007, Futohi et al., 2015, Acar et al., 2014, Bal et al., 2013, Chen et al., 2001). Importantly, pregnancy is considered a semi-allograft as the fetus can have HLA Ags that the mother does not have. The maternal immune system may recognize these Ags of paternal origin, as it has been demonstrated both in the maternal peripheral blood and in the placenta (Tilburgs et al., 2009, van Kampen et al., 2001). During pregnancy exchange of cells between mother and fetus will result in microchimerism (Maloney et al., 1999, Bianchi et al., 1996). The microchimeric cells carry Ags that the recipient does not have, which may result in sensitization of the mother or tolerance in the fetus (Bracamonte-Baran and Burlingham, 2015). However, different regulatory mechanisms are in place to prevent an allo-reactive immune response and create a tolerogenic environment for the undisturbed fetal development. For instance, decidual T cell activation is associated with a concomitant induction of functional T regulatory cells (Tilburgs et al., 2009). Furthermore, the extravillous trophoblasts do not express HLA-A, -B, -DR, -DQ and -DP (Moffett-King, 2002), but only HLA-C and non-classical HLA-E and -G.

The role of maternal and child HLA has mainly been shown in pregnancy complications such as pre-eclampsia, intrauterine growth retardation and recurrent spontaneous abortion (Faridi and Agrawal, 2011, Hiby et al., 2004), whereas its role during cCMV is unknown. When cCMV occurs,

the placental tolerogenic environment may be altered, resulting in an enhanced alloreactivity (Tilburgs and Strominger, 2013), which in turn may affect cCMV outcome. The aim of this study was to assess the maternal and child HLA molecules in relation to cCMV, neonatal viral load and clinical outcome from birth till 6 years of age.

6.2. MATERIALS AND METHODS

6.2.1. Study population and clinical data

A previously described nationwide, retrospective cohort was used in this study (Korndewal et al., 2015b). The cohort was derived from a total group of 31,484 children, born in 2008 in the Netherlands, which was retrospectively tested for cCMV infection by PCR of CMV DNA in neonatal dried blood spot (DBS) at five years of age. In total, 156 children (0.5%) were diagnosed with cCMV. After approval by the Medical Ethics Committee of the Leiden University Medical Center, the parents of 125 congenitally CMV infected children and of 263 non-infected children gave written informed consent for the retrieval of clinical data. For this study, 104 children with cCMV and their mothers additionally provided buccal swabs for HLA typing. Two buccal swabs were retrieved from each subject (FLOQSwabs hDNA Free, 20-mm breaking point in 174.5mm long dry tube, COPAN ITALIA SPA, Brescia, Italy). Children were defined as symptomatic at birth if they had one or more of the following signs or symptoms in the neonatal period: prematurity, being small for gestational age, microcephaly, hepato- or splenomegaly, generalized petechiae or purpura, hypotonia, abnormal laboratory findings (e.g. neutropenia or thrombocytopenia), cerebral ultrasound abnormalities, ophthalmologic abnormalities or neonatal hearing impairment. LTI was defined as the presence of impairment in one or more domains (hearing, visual, neurological, motor, cognitive and speech-language). The cCMV associated LTI in the original cohort has been described in detail (Korndewal et al., 2017). In brief, hearing impairment was defined as sensorineural hearing loss ≥ 40 dB; visual impairment was defined as a visual acuity below 0.3; neurological impairment included cerebral palsy, epilepsy, microcephaly, autism spectrum disorder and ADHD; motor developmental delay was based upon the physical therapist's report and if available on a score below the fifth centile in the Movement Assessment Battery for Children; cognitive developmental delay was defined as an intelligence quotient less than or equal to 70 if this was tested, or it was based on a diagnosis by a medical specialist; speech and language development were assessed by the speech therapist or speech and hearing centre. In this cohort maternal seroimmunity to CMV before birth was unknown, hence it was assumed that cCMV could have resulted from either maternal primary or secondary infection.

6.2.2. DNA extraction from buccal swabs and HLA typing

DNA was extracted from the buccal swabs by using QIAamp DNA (blood) mini kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction with minor modifications. The buccal swabs were incubated with PBS for 30 minutes at room temperature. The pre-incubation fluid was applied to one QIAamp spin column and eluted in 150 µl of Tris-EDTA-4 buffer. All mothers and children were DNA typed for the loci HLA-A (20 alleles), HLA-B (40 alleles), HLA-C (14 alleles),

HLA-DR (14 alleles) and HLA-DQ (7 alleles). For class I, a commercially available assay was applied, LIFECODES HLA-A, B and C SSO Typing kits (Immucor, Norcross, GA, US). Data were analysed using MatchIT software (Immucor-Lifecodes). For class II, a locally developed SSO technique was used as previously described (Verduyn et al., 1993), and data were analysed using SCORE software (Wolfgang-Helmberg).

6.2.3. DNA extraction from DBS and qPCR of CMV

After a first initial CMV PCR screening performed at the National Institute for Public Health and the Environment, a second confirmatory PCR was performed at the Leiden University Medical Center (Korndewal et al., 2015b). DNA was extracted from DBS by using the QIAamp DNA minikit according to the previously described protocol (de Vries et al., 2012). For each test one full DBS was punched by using an automated DBS puncher (1296-071, Perkin Elmer-Wallac, Zaventem, Belgium). CMV DNA amplification of a 126-bp fragment from the immediate-early antigen region was performed using an internally controlled quantitative real-time PCR, as described previously (de Vries et al., 2009, Kalpoe et al., 2004), on the CFX96 Real-Time PCR Detection System (BioRad, Veenendaal, The Netherlands). The PCR was performed in triplicate, and the viral load expressed in IU/ml.

6.2.4. Statistical analysis

Data were analyzed by using the Statistical Package for Social Sciences (SPSS, version 23, Chicago, IL, USA). The frequency of the HLA alleles in the study group was compared to the frequency in 5604 healthy Caucasian Dutch blood donor by using the two-sided Fisher's exact test (van Rooijen et al., 2012). The p-values were corrected for multiple comparisons (pm) conform to the Šidák method (Sidák, 1967). Odds ratios and corresponding 95% confidence intervals were calculated according to the Woolf Haldane test (Haldane, 1956, Woolf, 1955). A large control group could lead to significant differences which are clinically irrelevant. Therefore p-values were standardized (ps) to a smaller sample size following the method of Good (Good, 1982). Next, the study group was split into two groups according to the median viral load measured on DBS, namely high and low viral load group. The choice of the median was dictated by the study design, which involved several HLA molecules potentially leading to lack of statistical power, not allowing further division of the total group, and by the fact that there is no commonly accepted cut-off to define high and low viral load. The frequency of the HLA alleles in the high viral load group was compared to the frequency in the low viral load group by using the same method without correction for the sample size, which was similar in both groups. A Mann-Whitney test was used to further assess the distribution of viral load in relation to the HLA alleles of interest. Next, the maternal and child HLA allele frequencies were tested for Hardy-Weinberg Equilibrium (HWE), stating that in absence of other influences the genotype frequency in a certain population remains constant from generation to generation (Hardy, 1908). This has been suggested as a measure of disease association when analysing the case group *per se* as, by definition, the control group has to hold the HWE (Namipashaki et al., 2015, Lee, 2003, Nielsen et al., 1998). The HWE was assessed for both maternal and child genotypes by using Pypop software 0.7.0. The chi-square test was used to evaluate the observed and expected number

of HLA mismatches (mm), and non-inherited maternal antigens (NIMAs), in relation to symptoms at birth, LTI and viral load. With expected low values, a Fischer's exact test was used instead. A p-value < 0.05 was considered statistically significant.

6.3. RESULTS

6.3.1. Study population and clinical data

A total of 96 mother-child pairs were successfully typed for at least one of the following HLA alleles, HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ. Eight mother-child pairs could not be typed because of lack of DNA quality and concentration. The clinical data of the congenitally infected children included in this study are shown in Table 1. Nineteen (20%) children had symptoms at birth. Of these children, 11 (58%) had LTI at the age of 6 years. Additionally, 16 (21%) asymptomatic children had LTI. Overall, 27 (28%) of the total group of children with cCMV developed any LTI. The control group consisted of 5604 randomly selected healthy Dutch blood donors who were previously genotyped (van Rooijen et al., 2012). In the control group, which was in HWE, 3318 (59%) were females and the mean (SD) age was 36.2 (8.7) years. No additional clinical information nor CMV serostatus were available. Therefore, it was assumed that 50% was CMV-positive at the moment of typing (Korndewal et al., 2015a). This group is considered to be a proper representation of the HLA gene distribution in the Dutch population (Schipper et al., 1996).

Table 1. Characteristics and clinical outcome of study population

	Congenital CMV infection		
	n = 96	Asympt.[1] n = 77	Sympt.[2] n = 19
Gender			
Male	57	44	13
Female	39	33	6
Gestational age (weeks)[3]	39 (28-42)	39 (37-42)	36 (28-41)
Birth weight (g)[3]	3435 (900-5110)	3540 (2635-5110)	2800 (900-4170)
Long term impairment			
Hearing impairment[4]	3	2	1
Visual impairment[5]	2	2	0
Neurological impairment[6]	5	2	3
Motor impairment[7]	13	9	4
Cognitive impairment[8]	7	4	3
Speech/language problem[9]	18	10	8
One or more impairment[10]	27	16	11

[1] Asymptomatic at birth; [2] Symptomatic at birth: premature (n=11), dysmature (n=2); microcephaly (n=5); neonatal hearing loss (n=1); abnormal cranial ultrasound (n=1) [3] Median and range; [4] sensorineural hearing loss; [5] optic nerve atrophy (n=1), cortical visual impairment (n=1); [6] cerebral palsy (n=1), epilepsy (n=1), microcephaly (n=1), ADHD (n=1), autism (n=3); [7] motor impairment (fine, gross or balance) based on test or diagnosis or sensory processing disorder or developmental coordination disorder; [8] cognitive impairment based on test or diagnosis; [9] language impairment based on test or diagnosis, speech-impairment, oral motor skill difficulties or auditory processing disorder; [10] Any long-term impairment, in one or more domains; [11] Impairment in two or more domains.

6.3.2. Analysis of maternal and child genotype frequencies

First, we evaluated whether any HLA alleles included in this study was associated with cCMV. For this purpose, the frequency of HLA Class I (A, B and C) and Class II (DR and DQ) alleles were analysed in 96 mother-child pairs, and compared to the HLA frequencies in 5604 Dutch healthy blood donors. Data are reported in Table S1 in Supplementary material. Initial uncorrected association analysis revealed an increased frequency of HLA-B*39 and HLA-DRB1*12 compared to the control group in the mothers (10.4% and 4.1% p = 0.0066 for HLA-B*39; 9.6% and 3.7% p = 0.0097 for HLA-DRB1*12). Whereas, in the children, an increased frequency of HLA-C*02 and HLA-DRB1*12, and a decreased frequency of HLA-C*16, compared to the control group were observed (17.9% and 10.3% p = 0.0254 for HLA-C*02; 9.6% and 3.7% p = 0.0097 for HLA-DRB1*12; 1.1% and 6.1% p = 0.0456 for HLA-C*16). After correction, none of the aforementioned associations remained significant.

Next, the study group was evaluated in more details, and the genotype frequencies of HLA-A, HLA-B, HLA-C, HLA-DR and HLA-DQ from the mothers and their children were tested for the HWE. There was not enough discriminative power to determine if in the total group of alleles the HWE was maintained, both in the mothers and in the children. However, for HLA-DQ alleles, which are less polymorphic, the HWE could be assessed. For the mother, no deviations from the HWE were observed, whereas for the children a deviation was reported (Table 2). Additionally, all homozygote and heterozygote alleles for all loci were in HWE for both mothers and children (Table 2).

Finally, in order to evaluate whether any HLA allele included in this study was associated with CMV viral load, we split our study group in two according to the median viral load measured on DBS, which was 3.2 log (IU/ml), namely low (n = 47) and high viral load group (n = 48). The frequency of the HLA alleles of the high viral load group was compared to the frequency of the low viral load group, both in the mothers and in the children. In our cohort, the maternal viral load could not be assessed as maternal samples during pregnancy were not available, therefore the maternal HLA frequency was assessed only in relation to the child CMV viral load. Initial uncorrected association analysis revealed a few correlations (Table S2 in Supplementary material). In the mothers, HLA-C*03 was found increased in the high viral load group compared to the low (42.6% and 21.3% respectively, p = 0.0455). In the children, HLA-DRB1*04 was found decreased in the high viral load group compared to the low (10.6% and 37.0% respectively, p = 0.0034), as well as HLA-DQ8 (10.6% and 30.4% respectively, p = 0.0218). However, given the high linkage between HLA-DR4 and HLA-DQ8, the increase of HLA-DQ8 percentage in the high viral load group may be considered as a secondary effect to the increase of HLA-DRB1*04. After correction for multiple comparison, only HLA-DRB1*04 remained significant (p = 0.0401) (Table S2 in Supplementary material). Therefore, a Mann-Whitney test was used to further assess the distribution of viral load between HLA-DRB1*04 positive and HLA-DRB1*04 negative children. The median viral load was significantly lower in the HLA-DRB1*04 positive children compared to the HLA-DRB1*04 negative children (p = 0.017), whereas this was not observed in the mothers (p = 0.627) (Fig. 1). Additionally, HLA-DRB1*04 was evaluated in relation to symptoms at birth and LTI, and no associations were found (data not shown). Furthermore, as several evidences have been supporting a protective role of HLA-B*51 during infections, we wondered whether the same trend could be observed in the context of cCMV. In the children, HLA-B*51 frequency was increased in the low viral load group compared to the high viral load

Table 2. Hardy-Weinberg Equilibrium for HLA-Class I and HLA-Class II

	Mothers		Children	
	Chi-square	p-value	Chi-square	p-value
HLA-A				
total[1]	N/A[4]	N/A	N/A	N/A
homozygosity[2]	0.99	0.319	1.15	0.283
heterozygosity[3]	0.20	0.654	0.23	0.632
HLA-B				
total	N/A	N/A	N/A	N/A
homozygosity	0.86	0.355	0.13	0.718
heterozygosity	0.07	0.787	0.01	0.912
HLA-C				
total	N/A	N/A	N/A	N/A
homozygosity	0.21	0.644	0.18	0.672
heterozygosity	0.04	0.843	0.04	0.845
HLA-DR				
total	N/A	N/A	N/A	N/A
homozygosity	0.01	0.913	0.76	0.382
heterozygosity	0.00	0.969	0.10	0.749
HLA-DQ				
total	0.88	0.830	8.80	0.032*
homozygosity	0.18	0.675	0.00	1.000
heterozygosity	0.04	0.837	0.00	1.000

[1] Hardy-Weinberg Equilibrium for all HLA-A allele frequencies included in this study; [2] Hardy-Weinberg Equilibrium for the HLA-A homozygous alleles; [3] Hardy-Weinberg Equilibrium for the HLA-A heterozygous alleles; [4] N/A = not assessable, due to lack of statistical power the HWE could not be assessed. * significant p-value.

group (17.4% and 4.3% respectively, p = 0.050, pm = 0.694) (Table S2 in Supplementary material), and the median neonatal viral load was slightly lower in HLA-B*51 positive children compared to the HLA-B*51 negative children (p = 0.220) (Fig 2). These trends were not observed in the mothers (Table S2 in Supplementary material and Fig. 2). Finally, the frequency of the HLA alleles in relation to symptoms at birth (n = 19) and LTI (n = 27) could not be addressed due to the lack of statistical power.

6.3.3. Maternal-fetal HLA Class I and Class II mismatches in relation to cCMV clinical outcome

We next investigated whether maternal-fetal HLA mm were associated with a worse cCMV outcome. This was evaluated for HLA-A, HLA-B, HLA-DR and HLA-DQ as for HLA-C was already addressed (Rovito, 2018). For this purpose, mm were calculated on the basis of the presence of an Ag in the fetus, which was absent in the mother because the inherited paternal antigen was different from both maternal alleles (Table 3). The percentage of mm were compared between children symptomatic and asymptomatic at birth, as well as between children who developed LTI and those who did not. No differences in percentage of mm were observed in relation to symptoms at birth nor to LTI development (Table 4). Mm were additionally evaluated in relation to CMV viral

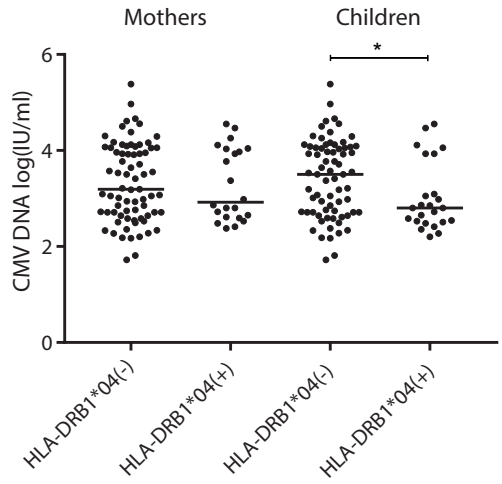
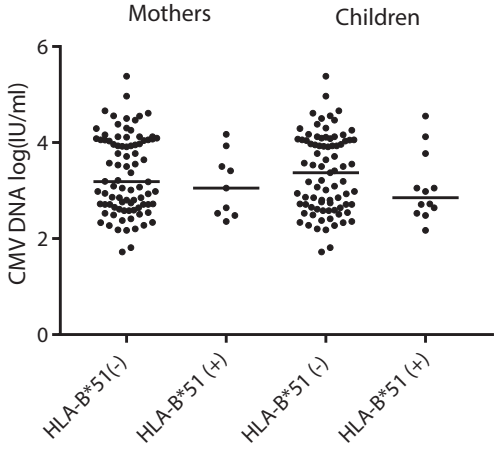


Figure 1. Maternal and child HLA-DRB1*04 in relation to neonatal viral load measured in DBS (log(IU/ml)). *Horizontal bars represent medians.* *p=0.017.



Figur 2. Maternal and child HLA-B*51 in relation to neonatal viral load measured in DBS (log(IU/ml)). *Horizontal bars represent medians.*

load, by comparing mm percentage between low and high viral load groups, both in the mothers and in the children. No difference in the mm percentage were observed between high and low viral load groups (data not shown).

6.3.4. Non-inherited maternal HLA Class I and HLA Class II in relation to cCMV clinical outcome

We finally assessed whether NIMAs influenced cCMV clinical outcome. This was evaluated for HLA-A, HLA-B, HLA-DR and HLA-DQ as for HLA-C was already addressed (Rovito, 2018). For

Table 3. Definitions of mismatches and NIMAs.

Genotype combinations[1]			
Maternal genotype	**Fetal genotype**	**mm[2]**	**NIMAs[3]**
a/b	a/a	no	yes
a/b	b/b	no	yes
a/b	a/c	yes	yes
a/b	c/b	yes	yes
a/a	a/b	yes	no
b/b	a/b	yes	no
a/a	a/a	no	no
a/b	a/b	no	no
b/b	b/b	no	no

[1] Combinations of maternal and child genotype by using 3 hypothetical genes (a, b, c); [2] mm: mismatches, defined as the Ag that the child has but the mother does not have, because the inherited paternal antigen is different from both maternal alleles; [3] NIMAs: non-inherited maternal antigens, defined as the Ag that the mother has and the child does not because the inherited paternal antigen differs from the non-inherited maternal antigen.

Table 4. HLA mismatches and NIMAs in relation to cCMV clinical outcome.

	Symptoms at birth			**Long-term impairments (LTI)**		
	Sympt.[1] **% n = 19**	**Asympt.[2]** **% n = 76[5]**	**p-value Chi**	**LTI (≥ 1)[3]** **% n = 26[6]**	**No LTI[4]** **% n = 69[7]**	**p-value Chi**
HLA-A mm[8]	78.9	64.5	0.229	76.9	63.8	0.223
HLA-B mm	94.7	80	0.179~	88.5	80.9	0.543~
HLA-DR mm	63.2	80	0.137~	68	79.7	0.236
HLA-DQ mm	47.4	64	0.185	52	63.8	0.302
HLA-A NIMAs[9]	89.5	72.4	0.145~	80.8	73.9	0.487
HLA-B NIMAs	84.2	88	0.703~	88.5	86.8	1.000~
HLA-DR NIMAs	78.9	80	1.000~	80	79.7	0.975
HLA-DQ NIMAs	63.2	57.3	0.645	56	59.4	0.766

[1] Symptomatic at birth; [2] Asymptomatic at birth; [3] Any long-term impairment, in one or more domains of impairments: hearing, visual neurologic, motor, cognitive, and speech-language; [4] Absence of any long-term impairment; [5] HLA-B, HLA-DR and HLA-DQ n = 75; [6] HLA-DR and HLA-DQ n = 25; [7] HLA-B n = 68; [8] mm = mismatches, defined as the Ag that the child has but the mother does not have because the inherited paternal antigen differed from both maternal alleles; [9] NIMAs = non-inherited maternal antigens, defined as the Ag in the mother which was absent in the child because the inherited paternal antigen differed from the NIMAs. ~ Fischer's exact test.

this purpose, NIMA was defined as an Ag in the mother, which was absent in the child because the inherited paternal antigen was different from the NIMA (Table 3). The percentage of NIMAs were compared between children symptomatic and asymptomatic at birth, as well as between children who developed LTI and those who did not. In our cohort, no differences in percentage of NIMAs were observed in relation to symptoms at birth nor to LTI development (Table 4). NIMAs

were additionally assessed in relation to CMV viral load, but no differences were observed between high and low viral load groups (data not shown). Interestingly, HLA-DR4 has been associated with genetic susceptibility to rheumatoid arthritis, and, in HLA-DR4 negative patients, HLA-DR4 NIMAs was found increased, suggesting that the maternal presence of this allele may have a role (ten Wolde et al., 1993). Therefore, we wondered whether, in HLA-DRB1*04 negative infected children, HLA-DRB1*04 NIMAs could still influence CMV viral load (Fig. S1 in Supplementary material). However, no NIMAs effect was observed in our cohort (p = 0.486), suggesting that the protective effect of HLA-DRB1*04 is driven by the presence of this allele in the child. Finally, HLA-DRB1*04 NIMAs were not associated with symptoms at birth nor to LTI (data not show).

6.4. DISCUSSION

This study aimed to determine whether certain maternal and child HLA alleles play a role in cCMV. Therefore, HLA of Class I and Class II were assessed in relation to cCMV and cCMV clinical outcome in a large retrospective nation-wide cohort of children with cCMV.

The analysis of maternal and child genotype frequencies in relation to cCMV, which was performed comparing the HLA alleles frequencies to the frequencies in a control group of healthy blood donors, did not show any striking association with cCMV. However, for the purpose of this study different control groups may be needed. From the maternal point of view, a group of CMV infected women who did not transmit the virus to the fetus would be more appropriate. Whereas from the neonatal point of view, a group of neonates that was exposed to CMV but did not get infected would be more suitable. This can be quite difficult to achieve as these HLA studies require large study groups.

For the aforementioned reasons, we looked in more details into our study group by first evaluating the HWE, and then the HLA frequencies in relation to neonatal viral load. The HWE could be determined only for the less polymorphic HLA-DQ, which was not in HWE in the group of children. Whether HLA-DQ has a role in cCMV would need to be further assessed as it has shown to have a minor function in our cohort. Additionally, in the children, HLA-DRB1*04 appeared to have a protective role during cCMV as its frequency was increased in the low viral load group compared to the high viral load group, and the median neonatal viral load was lower in HLA-DRB1*04 positive children (Table S2 IN Supplementary material and Fig 1). This remained significant even after statistical correction. Interestingly, HLA-DR4 has been associated with genetic susceptibility to rheumatoid arthritis, and, in HLA-DR4 negative patients, HLA-DR4 NIMAs was found increased (ten Wolde et al., 1993). However, in our cohort, HLA-DRB1*04 NIMAs did not influence CMV viral load. Therefore, the protective effect of HLA-DRB1*04 is most likely driven by the presence of the allele in the children (Fig. S1 in Supplementary material). Furthermore, HLA-DRB1*04 was not associated with clinical outcome, suggesting that it may have a specific role in the viral control rather than CMV-related disease. Consequently, it may be assumed a similar trend of that observed between child HLA-DRB1*04 and neonatal viral load, if maternal viral load during pregnancy was available. Importantly, the impact of HLA-DRB1*04 in CMV infection has already been shown. In allogeneic hematopoietic stem cell transplantation, CMV reactivation was less frequent in HLA-

DRB1*04 positive patients (Acar et al., 2014), and in kidney transplantation this allele protected from HBV, HCV and CMV infections (Gharesi-Fard et al., 2014). Additionally, as several evidences have been supporting a protective role of HLA-B*51 during infections, we wondered whether the same trend could be observed in the context of cCMV. Indeed, in kidney and allogeneic stem cell transplantation, HLA-B*51 has been shown to have a protective role towards CMV infection (Bal et al., 2013, Chen et al., 2001), while in infections, such as HIV, it was associated with an efficient immune control (Zhang et al., 2011). In our cohort, HLA-B*51 appeared to have a trend towards a better viral control, as its frequency was increased in the children of the low viral load group (Fig. 2). However, to confirm a possible protective role of HLA-DRB1*04 and HLA-B*51 more studies in other cohort of congenitally infected children would be needed. In our cohort, CMV viral load was not correlated to symptoms at birth nor to LTI development at 6 years of age (Rovito et al., 2017b, Rovito et al., 2017a). However, the predictive role of CMV viral load in blood for congenital CMV disease may differ depending on the timing of infection and whether there was a primary or recurrent maternal infection, which cannot be established in our cohort. Additionally, this cohort study, retrieved from a large population screening, does reflect a real population of newborns with cCMV in all its diversity, ranging from no symptoms at birth and no LTI to symptoms at birth with severe LTI. Therefore, the predictive value of CMV viral load could not be assessed for individual clinical outcomes. Importantly, the correlation between CMV viral load and clinical outcome has not been established yet as some studies have shown a correlation between CMV viral load and clinical outcome (Lanari et al., 2006, Forner et al., 2015), whereas others have not (Halwachs-Baumann et al., 2002, Binda et al., 2010, Ross et al., 2009).

Although the majority of the HLA molecules included in this study are not expressed at the placenta, an allogeneic response has been shown to occur due to the fetal microchimerism (Payne, 1957, van Kampen et al., 2001, van Kampen et al., 2002, Verdijk et al., 2004). Viral infections can increase the levels of pro-inflammatory cytokines and chemokines at the maternal-fetal interface, and intensify the decidual T cells influx (Constantin et al., 2007, Nancy et al., 2012). Here, the increased level of allogeneic maternal cells specific for fetal Ag that are not expressed by the trophoblast, may contribute to placental immunopathology by increasing the inflammation. The role of HLA mm in CMV infection has mainly been shown in transplantation. In allogeneic hematopoietic stem cell transplantation, CMV DNAemia, CMV disease and CMV resistance were associated with the presence of HLA mm (Sedky et al., 2014, Schonberger et al., 2010). Additionally, in kidney transplantation HLA mm were shown to be an important determinant of CMV-associated graft loss (Gatault et al., 2013). In the context of cCMV, HLA-A, HLA-B, HLA-DR and HLA-DQ mm, as well as NIMAs, were not significantly associated with clinical outcome, nor with CMV viral load. Even though the sample size may be a limiting factor in determining any significant HLA association, the HLA molecules expressed at the placenta, which are HLA-C, HLA-E and HLA-G, may exert a more important role in cCMV (Rovito, 2018). Interestingly, in uncomplicated pregnancies a significant correlation between the total number of HLA mm and an increased number of activated decidual T cells was reported (Tilburgs et al., 2009). However, HLA-C mm were the most influential in determining such an increase, whereas the other HLA mm contributed to a lesser degree (Tilburgs

et al., 2009). In our cohort, a slightly higher percentage of HLA-A and HLA-B mm were observed in the symptomatic group compared to the asymptomatic group, though not significant (Table 4).

Finally, some limitations characterise this study. First of all, potential effects of CMV on the expression of the HLA molecules included in this study could not be addressed. Indeed, CMV has developed strategies to evade host immunity and to establish latency, e.g. by down-regulating classical HLA molecules and up-regulating non-classical HLA (Gong et al., 2012, Onno et al., 2000, Huard and Fruh, 2000, Wilkinson et al., 2008). This could further influence the degree of HLA association with cCMV and cCMV clinical outcome. Second of all, the relatively small sample size may have limited the statistical power to detect any significant association. Third, due to the retrospective design of the study, cCMV diagnosis was performed by DBS testing, which in comparison with urine or saliva has been associated with limited sensitivity in some studies (Ross et al., 2014, Boppana et al., 2010, Inoue and Koyano, 2008). Urine specimens are commonly used for detection of cCMV due to the high viruria observed in congenitally infected infants, and this is still considered the gold standard method (Halwachs-Baumann et al., 2002). Urine and saliva are considered equally reliable for detection of cCMV (Yamamoto et al., 2006), and CMV viral load is usually lower in blood than in urine (Halwachs-Baumann et al., 2002). DBS can be considered a proper and reliable alternative specimen to fresh blood. Indeed, a positive correlation between CMV viral load measured on DBS and on whole blood, or on plasma, both in the context of cCMV and CMV in transplantation, has been previously shown (Christoni et al., 2012, Limaye et al., 2013). Importantly, even considering the relative reduction of CMV viral load on DBS during storage (Christoni et al., 2012), the specimens that could be potentially more affected from the aforementioned differences between blood and urine, or saliva, are those with low viral load. However, it is important to note that with the high sensitivity of our CMV PCR on DBS (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (Korndewal et al., 2015b). Therefore, the influence of the sensitivity of the CMV PCR on DBS on our conclusions can be considered negligible. Finally, the DBS specimens are available, almost, worldwide because they are collected for the screening of rare genetic metabolic disorders. This allowed us to perform a large-scale retrospective study for long-term cCMV outcome, which would be challenging in a prospective setting.

In conclusion, although none of the HLA alleles could be put forward as prognostic marker for cCMV or cCMV clinical outcome, our findings give useful insights into cCMV pathogenesis, and identify HLA-DRB1*04 and HLA-B*51 as potential HLAs correlating with a better viral control. Importantly, in view of the virus-host interaction at the maternal-fetal interface, the HLA expressed at the placenta may have a more substantial role in cCMV and cCMV clinical outcome (Rovito, 2018).

6.5. CONFLICT OF INTEREST STATEMENT

The authors declare no competing interests.

6.6. FUNDING

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SUPPORTING DATA

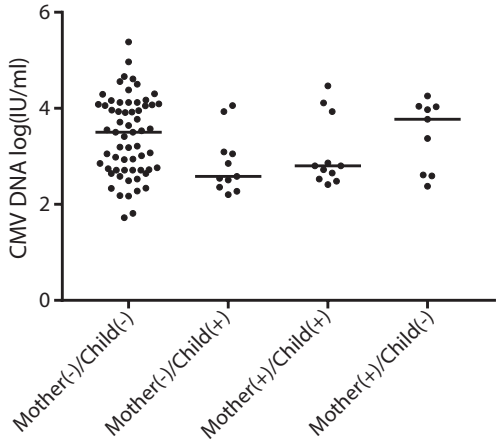

Figure S1. Maternal-child HLA-DRB1*04 combinations in relation to neonatal viral load measured in DBS (log(IU/ml)). Mother(-)/Child(-): mother and child HLA-DRB1*04 negative; Mother(-)/Child(+): mother HLA-DRB1*04 negative and child HLA-DRB1*04 positive; Mother(+)/Child(+): mother and child HLA-DRB1*04 positive; Mother(+)/Child(-): mother HLA-DRB1*04 positive and child HLA-DRB1*04 negative. *Horizontal bars represent medians.*

Table S1. HLA frequencies of mothers and children compared to the control group

	Mothers[1]			Controls[4]			Odds ratio and 95% C.I.[7]			Fisher's Exact[8]		
Antigen	Pos[2]	Neg[3]		Pos[5]	Neg[6]	%	OR	Lower	Upper	P[8]	Pm[9]	Ps[10]
A*01	29	66	30.5	1887	3717	33.7	0.87	0.564	1.353	0.5845	1.0000	0.5000
A*02	51	44	53.7	2745	2859	49.0	1.21	0.804	1.806	0.4080	1.0000	0.5000
A*03	26	69	27.4	1640	3964	29.3	0.92	0.587	1.447	0.7341	1.0000	0.5000
A*11	13	82	13.7	649	4955	11.6	1.25	0.698	2.233	0.5175	1.0000	0.5000
A*23	1	94	1.1	134	5470	2.4	0.65	0.128	3.269	0.7285	1.0000	0.5000
A*24	21	74	22.1	921	4683	16.4	1.47	0.903	2.382	0.1619	0.9708	0.5000
A*25	1	94	1.1	122	5482	2.2	0.71	0.140	3.600	0.7244	1.0000	0.5000
A*26	4	91	4.2	248	5356	4.4	1.06	0.408	2.755	1.0000	1.0000	0.5000
A*29	6	89	6.3	299	5305	5.3	1.29	0.575	2.877	0.6425	1.0000	0.5000
A*30	2	93	2.1	276	5328	4.9	0.52	0.146	1.820	0.3302	0.9997	0.5000
A*31	9	86	9.5	314	5290	5.6	1.85	0.936	3.645	0.1136	0.9103	0.5000
A*32	6	89	6.3	355	5249	6.3	1.07	0.480	2.395	1.0000	1.0000	0.5000
A*33	2	93	2.1	113	5491	2.0	1.29	0.363	4.605	0.7177	1.0000	0.5000
A*34	0	95	0.0	17	5587	0.3	1.67	0.100	28.002	1.0000	1.0000	0.5000
A*36	1	94	1.1	9	5595	0.2	9.35	1.651	52.947	0.1548	0.9654	0.5000
A*66	0	95	0.0	33	5571	0.6	0.87	0.053	14.316	1.0000	1.0000	0.5000
A*68	6	89	6.3	517	5087	9.2	0.71	0.320	1.591	0.4709	1.0000	0.5000
A*69	0	95	0.0	9	5595	0.2	3.08	0.178	53.368	1.0000	1.0000	0.5000
A*74	0	95	0.0	5	5599	0.1	5.33	0.293	97.084	1.0000	1.0000	0.5000
A*80	0	95	0.0	2	5602	0.0	11.73	0.559	246.054	1.0000	1.0000	0.5000

Table s1. (continued)

	Mothers[1]			Controls[4]			Odds ratio and 95% C.I.[7]			Fisher's Exact[8]		
Antigen	**Pos[2]**	**Neg[3]**	**%**	**Pos[5]**	**Neg[6]**	**%**	**OR**	**Lower**	**Upper**	**P[8]**	**Pm[9]**	**Ps[10]**
B*07	21	75	21,9	1391	4213	24,8	0,86	0,532	1,398	0,5530	1,0000	0,5000
B*08	21	75	21,9	1436	4168	25,6	0,83	0,510	1,339	0,4790	1,0000	0,5000
B*13	2	94	2,1	229	5375	4,1	0,62	0,175	2,191	0,4391	1,0000	0,5000
B14	3	93	3,1	217	5387	3,9	0,93	0,316	2,719	1,0000	1,0000	0,5000
B62	11	85	11,5	837	4732	15,0	0,76	0,409	1,413	0,3881	1,0000	0,5000
B63	0	96	0,0	44	5525	0,8	0,64	0,039	10,523	1,0000	1,0000	0,5000
B75	1	95	1,0	16	5553	0,3	5,29	0,982	28,470	0,2525	1,0000	0,5000
B76	0	96	0,0	1	5568	0,0	19,23	0,779	475,189	1,0000	1,0000	0,5000
B77	0	96	0,0	9	5560	0,2	3,03	0,175	52,480	1,0000	1,0000	0,5000
B*18	5	91	5,2	414	5190	7,4	0,75	0,317	1,790	0,5537	1,0000	0,5000
B*27	9	87	9,4	405	5199	7,2	1,39	0,707	2,740	0,4239	1,0000	0,5000
B*35	17	79	17,7	1090	4514	19,5	0,91	0,541	1,535	0,7946	1,0000	0,5000
B*37	2	94	2,1	181	5423	3,2	0,79	0,223	2,800	0,7708	1,0000	0,5000
B*38	4	92	4,2	186	5418	3,3	1,41	0,543	3,682	0,5625	1,0000	0,5000
B*39	**10**	**86**	**10,4**	**229**	**5375**	**4,1**	**2,84**	**1,478**	**5,468**	**0,0066**	**0,2325**	**0,5000**
B60	13	83	13,5	676	4832	12,3	1,15	0,646	2,064	0,6404	1,0000	0,5000
B61	7	89	7,3	198	5310	3,6	2,24	1,050	4,786	0,0880	0,9749	0,5000
B*41	1	95	1,0	86	5518	1,5	1,00	0,197	5,098	1,0000	1,0000	0,5000
B*42	1	95	1,0	9	5595	0,2	9,25	1,634	52,387	0,1563	0,9989	0,5000
B*44	28	68	29,2	1257	4347	22,4	1,44	0,925	2,237	0,1383	0,9974	0,5000
B*45	1	95	1,0	60	5544	1,1	1,44	0,281	7,366	1,0000	1,0000	0,5000
B*46	0	96	0,0	9	5595	0,2	3,05	0,176	52,811	1,0000	1,0000	0,5000
B*47	0	96	0,0	29	5575	0,5	0,98	0,059	16,144	1,0000	1,0000	0,5000
B*48	0	96	0,0	5	5599	0,1	5,28	0,290	96,071	1,0000	1,0000	0,5000
B*49	4	92	4,2	84	5520	1,5	3,18	1,205	8,386	0,0604	0,9171	0,5000
B50	1	95	1,0	84	5520	1,5	1,03	0,202	5,222	1,0000	1,0000	0,5000
B*51	9	87	9,4	590	5014	10,5	0,92	0,469	1,811	0,8668	1,0000	0,5000
B*52	3	93	3,1	50	5554	0,9	4,12	1,367	12,400	0,0593	0,9131	0,5000
B*53	0	96	0,0	40	5564	0,7	0,71	0,043	11,662	1,0000	1,0000	0,5000
B*54	0	96	0,0	2	5602	0,0	11,61	0,554	243,488	1,0000	1,0000	0,5000
B*55	2	94	2,1	239	5365	4,3	0,59	0,168	2,095	0,4404	1,0000	0,5000
B*56	1	95	1,0	77	5527	1,4	1,12	0,220	5,708	1,0000	1,0000	0,5000
B*57	8	88	8,3	368	5236	6,6	1,36	0,670	2,781	0,5298	1,0000	0,5000
B*58	1	95	1,0	93	5511	1,7	0,93	0,182	4,705	1,0000	1,0000	0,5000
B*67	0	96	0,0	1	5603	0,0	19,36	0,783	478,175	1,0000	1,0000	0,5000
B*71	0	96	0,0	19	5550	0,3	1,47	0,088	24,604	1,0000	1,0000	0,5000
B72	1	95	1,0	26	5543	0,5	3,29	0,626	17,235	0,3703	1,0000	0,5000
B*73	0	96	0,0	2	5602	0,0	11,61	0,554	243,488	1,0000	1,0000	0,5000
B*78	0	96	0,0	3	5601	0,1	8,29	0,425	161,645	1,0000	1,0000	0,5000
B*81	0	96	0,0	3	5601	0,1	8,29	0,425	161,645	1,0000	1,0000	0,5000
C*01	4	91	4.2	323	5281	5.8	0.80	0.310	2.082	0.6594	1.0000	0.5000
C*02	15	80	15.8	575	5029	10.3	1.68	0.970	2.918	0.0881	0.7249	0.5000
C*03	30	65	31.6	1665	3939	29.7	1.10	0.714	1.699	0.7343	1.0000	0.5000

Table s1. (continued)

	Mothers[1]			Controls[4]			Odds ratio and 95% C.I.[7]			Fisher's Exact[8]		
Antigen	**Pos[2]**	**Neg[3]**	**%**	**Pos[5]**	**Neg[6]**	**%**	**OR**	**Lower**	**Upper**	**P[8]**	**Pm[9]**	**Ps[10]**
C*04	18	77	18.9	1287	4317	23.0	0.80	0.480	1.334	0.3910	0.9990	0.5000
C*05	19	76	20.0	764	4840	13.6	1.61	0.976	2.669	0.0959	0.7563	0.5000
C*06	14	81	14.7	898	4706	16.0	0.93	0.531	1.637	0.8876	1.0000	0.5000
C*07	46	49	48.4	3140	2464	56.0	0.74	0.492	1.104	0.1455	0.8893	0.5000
C*08	3	92	3.2	233	5371	4.2	0.87	0.297	2.551	1.0000	1.0000	0.5000
C*12	13	82	13.7	466	5138	8.3	1.80	1.006	3.230	0.0890	0.7287	0.5000
C*14	2	93	2.1	117	5487	2.1	1.25	0.351	4.443	1.0000	1.0000	0.5000
C*15	7	88	7.4	287	5317	5.1	1.57	0.737	3.334	0.3422	0.9972	0.5000
C*16	4	91	4.2	343	5261	6.1	0.75	0.291	1.953	0.6623	1.0000	0.5000
C*17	2	93	2.1	97	5507	1.7	1.51	0.423	5.389	0.6813	1.0000	0.5000
C*18	0	95	0.0	2	5602	0.0	11.73	0.559	246.054	1.0000	1.0000	0.5000
DRB1*01	21	73	22,3	1234	4370	22,0	1,04	0,638	1,681	0,9007	1,0000	0,5000
DR17	24	70	25,5	1524	3990	27,6	0,91	0,572	1,446	0,7275	1,0000	0,5000
DR18	0	94	0,0	8	5506	0,1	3,43	0,196	59,819	1,0000	1,0000	0,5000
DRB1*04	20	74	21,3	1557	4047	27,8	0,72	0,437	1,170	0,2004	0,9563	0,5000
DRB1*07	16	78	17,0	1098	4506	19,6	0,86	0,505	1,472	0,6013	1,0000	0,5000
DRB1*08	3	91	3,2	361	5243	6,4	0,55	0,190	1,622	0,2845	0,9908	0,5000
DRB1*09	1	93	1,1	160	5444	2,9	0,54	0,108	2,752	0,5242	1,0000	0,5000
DRB1*10	2	92	2,1	132	5472	2,4	1,12	0,314	3,967	1,0000	1,0000	0,5000
DRB1*11	22	72	23,4	930	4674	16,6	1,56	0,966	2,515	0,0929	0,7448	0,5000
DRB1*12	**9**	**85**	**9,6**	**210**	**5394**	**3,7**	**2,85**	**1,436**	**5,645**	**0,0097**	**0,1273**	**0,4957**
DRB1*13	23	71	24,5	1393	4211	24,9	0,99	0,621	1,589	1,0000	1,0000	0,5000
DRB1*14	8	86	8,5	373	5231	6,7	1,38	0,675	2,806	0,4084	0,9994	0,5000
DRB1*15	25	69	26,6	1356	4248	24,2	1,15	0,727	1,816	0,6272	1,0000	0,5000
DRB1*16	3	91	3,2	165	5439	2,9	1,26	0,428	3,697	0,7569	1,0000	0,5000
DQ7	35	59	37,2	1641	3894	29,6	1,42	0,930	2,154	0,1124	0,5661	0,5000
DQ8	16	78	17,0	1073	4462	19,4	0,87	0,512	1,492	0,6926	0,9997	0,5000
DQ9	7	87	7,4	504	5031	9,1	0,85	0,403	1,812	0,7179	0,9999	0,5000
DQB1*02	33	61	35,1	2204	3400	39,3	0,84	0,550	1,284	0,4564	0,9860	0,5000
DQB1*04	3	91	3,2	340	5264	6,1	0,59	0,202	1,730	0,3770	0,9636	0,5000
DQB1*05	33	61	35,1	1840	3764	32,8	1,11	0,729	1,703	0,6584	0,9995	0,5000
DQB1*06	41	53	43,6	2470	3134	44,1	0,98	0,654	1,481	1,0000	1,0000	0,5000

	Children[11]			Controls			Odds ratio and 95% C.I.			Fisher's Exact		
Antigen	**Pos**	**Neg**	**%**	**pos**	**neg**	**%**	**OR**	**lower**	**upper**	**P**	**Pm**	**Ps**
A*01	31	64	32.6	1887	3717	33.7	0.96	0.626	1.478	0.9129	1.0000	0.5000
A*02	48	47	50.5	2745	2859	49.0	1.06	0.710	1.592	0.8362	1.0000	0.5000
A*03	23	72	24.2	1640	3964	29.3	0.78	0.490	1.252	0.3074	0.9994	0.5000
A*11	10	85	10.5	649	4955	11.6	0.94	0.491	1.788	0.8720	1.0000	0.5000
A*23	4	91	4.2	134	5470	2.4	2.00	0.765	5.233	0.2932	0.9990	0.5000
A*24	19	76	20.0	921	4683	16.4	1.30	0.784	2.141	0.3314	0.9997	0.5000
A*25	2	93	2.1	122	5482	2.2	1.20	0.336	4.256	1.0000	1.0000	0.5000

Table s1. (continued)

	Children[11]			Controls			Odds ratio and 95% C.I.			Fisher's Exact		
Antigen	**Pos**	**Neg**	**%**	**pos**	**neg**	**%**	**OR**	**lower**	**upper**	**P**	**Pm**	**Ps**
A*26	3	92	3.2	248	5356	4.4	0.82	0.278	2.389	0.7997	1.0000	0.5000
A*29	2	93	2.1	299	5305	5.3	0.47	0.134	1.672	0.2421	0.9961	0.5000
A*30	2	93	2.1	276	5328	4.9	0.52	0.146	1.820	0.3302	0.9997	0.5000
A*31	8	87	8.4	314	5290	5.6	1.63	0.801	3.335	0.2557	0.9973	0.5000
A*32	8	87	8.4	355	5249	6.3	1.43	0.704	2.924	0.3933	1.0000	0.5000
A*33	0	95	0.0	113	5491	2.0	0.25	0.016	4.105	0.2658	0.9979	0.5000
A*34	0	95	0.0	17	5587	0.3	1.67	0.100	28.002	1.0000	1.0000	0.5000
A*36	0	95	0.0	9	5595	0.2	3.08	0.178	53.368	1.0000	1.0000	0.5000
A*66	2	93	2.1	33	5571	0.6	4.45	1.210	16.336	0.1149	0.9129	0.5000
A*68	8	87	8.4	517	5087	9.2	0.96	0.470	1.942	1.0000	1.0000	0.5000
A*69	0	95	0.0	9	5595	0.2	3.08	0.178	53.368	1.0000	1.0000	0.5000
A*74	0	95	0.0	5	5599	0.1	5.33	0.293	97.084	1.0000	1.0000	0.5000
A*80	0	95	0.0	2	5602	0.0	11.73	0.559	246.054	1.0000	1.0000	0.5000
B*07	21	73	22,3	1391	4213	24,8	0,89	0,546	1,438	0,6316	1,0000	0,5000
B*08	22	72	23,4	1436	4168	25,6	0,90	0,559	1,451	0,7209	1,0000	0,5000
B*13	3	91	3,2	229	5375	4,1	0,90	0,306	2,627	1,0000	1,0000	0,5000
B14	2	92	2,1	217	5387	3,9	0,67	0,189	2,369	0,5864	1,0000	0,5000
B62	16	78	17,0	837	4732	15,0	1,19	0,695	2,030	0,5619	1,0000	0,5000
B63	2	92	2,1	44	5525	0,8	3,36	0,923	12,196	0,1771	0,9996	0,5000
B75	0	94	0,0	16	5553	0,3	1,78	0,106	29,903	1,0000	1,0000	0,5000
B76	0	94	0,0	1	5568	0,0	19,64	0,795	485,310	1,0000	1,0000	0,5000
B77	0	94	0,0	9	5560	0,2	3,10	0,179	53,599	1,0000	1,0000	0,5000
B*18	6	88	6,4	414	5190	7,4	0,92	0,412	2,053	0,8439	1,0000	0,5000
B*27	9	85	9,6	405	5199	7,2	1,42	0,723	2,806	0,4183	1,0000	0,5000
B*35	13	81	13,8	1090	4514	19,5	0,69	0,384	1,224	0,1895	0,9998	0,5000
B*37	2	92	2,1	181	5423	3,2	0,81	0,228	2,861	0,7704	1,0000	0,5000
B*38	4	90	4,3	186	5418	3,3	1,44	0,554	3,765	0,5562	1,0000	0,5000
B*39	3	91	3,2	229	5375	4,1	0,90	0,306	2,627	1,0000	1,0000	0,5000
B60	17	77	18,1	676	4832	12,3	1,61	0,954	2,727	0,1114	0,9911	0,5000
B61	6	88	6,4	198	5310	3,6	1,96	0,875	4,413	0,1566	0,9989	0,5000
B*41	0	94	0,0	86	5518	1,5	0,34	0,021	5,481	0,4053	1,0000	0,5000
B*42	0	94	0,0	9	5595	0,2	3,12	0,180	53,936	1,0000	1,0000	0,5000
B*44	24	70	25,5	1257	4347	22,4	1,20	0,755	1,911	0,4567	1,0000	0,5000
B*45	0	94	0,0	60	5544	1,1	0,48	0,030	7,900	0,6271	1,0000	0,5000
B*46	0	94	0,0	9	5595	0,2	3,12	0,180	53,936	1,0000	1,0000	0,5000
B*47	1	93	1,1	29	5575	0,5	3,03	0,580	15,839	0,3937	1,0000	0,5000
B*48	0	94	0,0	5	5599	0,1	5,39	0,296	98,119	1,0000	1,0000	0,5000
B*49	2	92	2,1	84	5520	1,5	1,77	0,494	6,316	0,6535	1,0000	0,5000
B50	0	94	0,0	84	5520	1,5	0,35	0,021	5,614	0,6500	1,0000	0,5000
B*51	10	84	10,6	590	5014	10,5	1,06	0,553	2,015	1,0000	1,0000	0,5000
B*52	0	94	0,0	50	5554	0,9	0,58	0,036	9,503	1,0000	1,0000	0,5000
B*53	0	94	0,0	40	5564	0,7	0,73	0,044	11,911	1,0000	1,0000	0,5000
B*54	0	94	0,0	2	5602	0,0	11,86	0,565	248,675	1,0000	1,0000	0,5000

Table s1. (continued)

	Children[11]			Controls			Odds ratio and 95% C.I.			Fisher's Exact		
Antigen	**Pos**	**Neg**	**%**	**pos**	**neg**	**%**	**OR**	**lower**	**upper**	**P**	**Pm**	**Ps**
B*55	3	91	3,2	239	5365	4,3	0,86	0,292	2,512	0,7987	1,0000	0,5000
B*56	1	93	1,1	77	5527	1,4	1,14	0,224	5,832	1,0000	1,0000	0,5000
B*57	10	84	10,6	368	5236	6,6	1,77	0,922	3,382	0,1377	0,9973	0,5000
B*58	1	93	1,1	93	5511	1,7	0,95	0,186	4,807	1,0000	1,0000	0,5000
B*67	0	94	0,0	1	5603	0,0	19,77	0,800	488,360	1,0000	1,0000	0,5000
B*71	0	94	0,0	19	5550	0,3	1,51	0,090	25,129	1,0000	1,0000	0,5000
B72	0	94	0,0	26	5543	0,5	1,11	0,067	18,297	1,0000	1,0000	0,5000
B*73	0	94	0,0	2	5602	0,0	11,86	0,565	248,675	1,0000	1,0000	0,5000
B*78	0	94	0,0	3	5601	0,1	8,47	0,434	165,090	1,0000	1,0000	0,5000
B*81	0	94	0,0	3	5601	0,1	8,47	0,434	165,090	1,0000	1,0000	0,5000
C*01	4	91	4.2	323	5281	5.8	0.80	0.310	2.082	0.6594	1.0000	0.5000
C*02	**17**	**78**	**17.9**	**575**	**5029**	**10.3**	**1.95**	**1.152**	**3.294**	**0.0254**	**0.3023**	**0.5000**
C*03	35	60	36.8	1665	3939	29.7	1.39	0.913	2.109	0.1416	0.8821	0.5000
C*04	19	76	20.0	1287	4317	23.0	0.85	0.518	1.411	0.5405	1.0000	0.5000
C*05	15	80	15.8	764	4840	13.6	1.22	0.704	2.111	0.5461	1.0000	0.5000
C*06	16	79	16.8	898	4706	16.0	1.09	0.637	1.856	0.7790	1.0000	0.5000
C*07	52	43	54.7	3140	2464	56.0	0.95	0.632	1.420	0.8352	1.0000	0.5000
C*08	2	93	2.1	233	5371	4.2	0.62	0.174	2.175	0.4390	0.9997	0.5000
C*12	9	86	9.5	466	5138	8.3	1.21	0.615	2.380	0.7063	1.0000	0.5000
C*14	1	94	1.1	117	5487	2.1	0.74	0.146	3.758	0.7242	1.0000	0.5000
C*15	4	91	4.2	287	5317	5.1	0.91	0.350	2.361	1.0000	1.0000	0.5000
C*16	**1**	**94**	**1.1**	**343**	**5261**	**6.1**	**0.24**	**0.048**	**1.224**	**0.0456**	**0.4794**	**0.5000**
C*17	0	95	0.0	97	5507	1.7	0.30	0.018	4.797	0.4124	0.9994	0.5000
C*18	0	95	0.0	2	5602	0.0	11.73	0.559	246.054	1.0000	1.0000	0.5000
DRB1*01	22	72	23,4	1234	4370	22,0	1,10	0,682	1,771	0,7083	1,0000	0,5000
DR17	22	72	23,4	1524	3990	27,6	0,81	0,504	1,308	0,4158	0,9995	0,5000
DR18	0	94	0,0	8	5506	0,1	3,43	0,196	59,819	1,0000	1,0000	0,5000
DRB1*04	22	72	23,4	1557	4047	27,8	0,81	0,501	1,299	0,4159	0,9995	0,5000
DRB1*07	22	72	23,4	1098	4506	19,6	1,27	0,790	2,053	0,3598	0,9981	0,5000
DRB1*08	4	90	4,3	361	5243	6,4	0,72	0,278	1,870	0,5242	1,0000	0,5000
DRB1*09	1	93	1,1	160	5444	2,9	0,54	0,108	2,752	0,5242	1,0000	0,5000
DRB1*10	2	92	2,1	132	5472	2,4	1,12	0,314	3,967	1,0000	1,0000	0,5000
DRB1*11	10	84	10,6	930	4674	16,6	0,62	0,327	1,190	0,1595	0,9121	0,5000
DRB1*12	**9**	**85**	**9,6**	**210**	**5394**	**3,7**	**2,85**	**1,436**	**5,645**	**0,0097**	**0,1273**	**0,4957**
DRB1*13	27	67	28,7	1393	4211	24,9	1,23	0,787	1,926	0,4003	0,9992	0,5000
DRB1*14	8	86	8,5	373	5231	6,7	1,38	0,675	2,806	0,4084	0,9994	0,5000
DRB1*15	25	69	26,6	1356	4248	24,2	1,15	0,727	1,816	0,6272	1,0000	0,5000
DRB1*16	0	94	0,0	165	5439	2,9	0,17	0,011	2,813	0,1160	0,8219	0,5000
DQ7	26	68	27,7	1641	3894	29,6	0,92	0,584	1,442	0,7334	0,9999	0,5000
DQ8	19	75	20,2	1073	4462	19,4	1,07	0,650	1,774	0,7937	1,0000	0,5000
DQ9	9	85	9,6	504	5031	9,1	1,11	0,563	2,180	0,8560	1,0000	0,5000
DQB1*02	36	58	38,3	2204	3400	39,3	0,96	0,634	1,460	0,9153	1,0000	0,5000

Table s1. (continued)

	Children[11]			Controls			Odds ratio and 95% C.I.			Fisher's Exact		
Antigen	Pos	Neg	%	pos	neg	%	OR	lower	upper	P	Pm	Ps
DQB1*04	4	90	4,3	340	5264	6,1	0,77	0,296	1,994	0,6605	0,9995	0,5000
DQB1*05	32	62	34,0	1840	3764	32,8	1,06	0,694	1,631	0,8250	1,0000	0,5000
DQB1*06	44	50	46.8	2470	3134	44.1	1.12	0.745	1.679	0.6021	0.9984	0.5000

[1] HLA alleles frequencies in the mothers; [2] Mothers positive for the specific HLA allele; [3] Mothers negative for the specific HLA allele; [4] Control group of 5604 randomly selected healthy Dutch blood donors; [5] Controls positive for the specific HLA allele; [6] Controls negative for the specific HLA allele; [7] Odds ratio (OR) and 95% confidence intervals (95% C.I.) calculated based on the Woolf-Haldane method; [8] Two-sided Fisher's exact test comparing HLA alleles frequencies between study and control group; [9] Pm: corrected p-values for multiple comparisons (Šidák method); [10] Ps: standardized p-values to a smaller sample size (Good method); [11] HLA alleles frequencies in the children.

Table S2. HLA frequencies of mothers and children in relation to neonatal viral load

	Mothers High viral load[1]			Mothers Low viral load[4]			Odds ratio and 95% C.I.[5]			Fisher's Exact[6]	
Antigen	Pos[2]	Neg[3]	%	pos	neg	%	OR[5]	lower	upper	P[6]	Pm[7]
A*01	11	36	23.4	18	29	38.3	0.50	0.208	1.213	0.1798	0.9489
A*02	26	21	55.3	24	23	51.1	1.18	0.530	2.638	0.8364	1.0000
A*03	14	33	29.8	12	35	25.5	1.23	0.504	2.999	0.8180	1.0000
A*11	8	39	17.0	5	42	10.6	1.66	0.523	5.287	0.5516	1.0000
A*23	0	47	0.0	1	46	2.1	0.33	0.013	8.217	1.0000	1.0000
A*24	12	35	25.5	9	38	19.1	1.43	0.547	3.723	0.6212	1.0000
A*25	0	47	0.0	1	46	2.1	0.33	0.013	8.217	1.0000	1.0000
A*26	2	45	4.3	2	45	4.3	1.00	0.165	6.053	1.0000	1.0000
A*29	3	44	6.4	3	44	6.4	1.00	0.215	4.659	1.0000	1.0000
A*30	0	47	0.0	2	45	4.3	0.19	0.009	4.100	0.4946	1.0000
A*31	2	45	4.3	7	40	14.9	0.30	0.067	1.320	0.1582	0.9244
A*32	3	44	6.4	3	44	6.4	1.00	0.215	4.659	1.0000	1.0000
A*33	2	45	4.3	0	47	0.0	5.22	0.244	111.718	0.4946	1.0000
A*36	0	47	0.0	1	46	2.1	0.33	0.013	8.217	1.0000	1.0000
A*68	3	44	6.4	3	44	6.4	1.00	0.215	4.659	1.0000	1.0000
B*07	10	38	20,8	10	37	21,3	0,97	0,371	2,560	1,0000	1,0000
B*08	9	39	18,8	12	35	25,5	0,68	0,262	1,780	0,4669	1,0000
B*13	1	47	2,1	1	46	2,1	0,98	0,098	9,755	1,0000	1,0000
B*18	3	45	6,3	2	45	4,3	1,40	0,262	7,469	1,0000	1,0000
B*27	4	44	8,3	5	42	10,6	0,78	0,210	2,910	0,7401	1,0000
B*35	8	40	16,7	9	38	19,1	0,85	0,305	2,371	0,7944	1,0000
B*37	1	47	2,1	1	46	2,1	0,98	0,098	9,755	1,0000	1,0000
B*38	1	47	2,1	3	44	6,4	0,40	0,057	2,839	0,3616	1,0000
B*39	3	45	6,3	7	40	14,9	0,42	0,109	1,583	0,1986	0,9980
B*41	1	47	2,1	0	47	0,0	3,00	0,119	75,524	1,0000	1,0000

Table S2. (continued)

	Mothers High viral load[1]			Mothers Low viral load[4]			Odds ratio and 95% C.I.[5]			Fisher's Exact[6]	
Antigen	Pos[2]	Neg[3]	%	pos	neg	%	OR[5]	lower	upper	P[6]	Pm[7]
B*42	0	48	0,0	1	46	2,1	0,32	0,013	8,046	0,4947	1,0000
B*44	14	34	29,2	13	34	27,7	1,07	0,446	2,586	1,0000	1,0000
B*45	0	48	0,0	1	46	2,1	0,32	0,013	8,046	0,4947	1,0000
B*49	1	47	2,1	3	44	6,4	0,40	0,057	2,839	0,3616	1,0000
B*51	4	44	8,3	5	42	10,6	0,78	0,210	2,910	0,7401	1,0000
B*52	3	45	6,3	0	47	0,0	7,31	0,367	145,464	0,2421	0,9996
B*55	0	48	0,0	2	45	4,3	0,19	0,009	4,015	0,2421	0,9996
B*56	1	47	2,1	0	47	0,0	3,00	0,119	75,524	1,0000	1,0000
B*57	4	44	8,3	4	43	8,5	0,98	0,248	3,854	1,0000	1,0000
B*58	1	47	2,1	0	47	0,0	3,00	0,119	75,524	1,0000	1,0000
B50	0	48	0,0	1	46	2,1	0,32	0,013	8,046	0,4947	1,0000
B60	9	39	18,8	4	43	8,5	2,33	0,699	7,731	0,2321	0,9994
B61	5	43	10,4	2	45	4,3	2,30	0,488	10,861	0,4353	1,0000
B62	8	40	16,7	3	44	6,4	2,67	0,716	9,945	0,1986	0,9980
B64	0	48	0,0	1	46	2,1	0,32	0,013	8,046	0,4947	1,0000
B65	1	47	2,1	1	46	2,1	0,98	0,098	9,755	1,0000	1,0000
B72	0	48	0,0	1	46	2,1	0,32	0,013	8,046	0,4947	1,0000
B75	1	47	2,1	0	47	0,0	3,00	0,119	75,524	1,0000	1,0000
C*01	2	45	4.3	2	45	4.3	1.00	0.165	6.053	1.0000	1.0000
C*02	8	39	17.0	7	40	14.9	1.16	0.396	3.406	1.0000	1.0000
C*03	20	27	42.6	10	37	21.3	2.66	1.091	6.495	0.0455	0.4543
C*04	10	37	21.3	8	39	17.0	1.30	0.475	3.568	0.7939	1.0000
C*05	7	40	14.9	11	36	23.4	0.59	0.211	1.635	0.4323	0.9994
C*06	6	41	12.8	8	39	17.0	0.73	0.240	2.209	0.7730	1.0000
C*07	22	25	46.8	23	24	48.9	0.92	0.413	2.049	1.0000	1.0000
C*08	1	46	2.1	2	45	4.3	0.59	0.074	4.630	1.0000	1.0000
C*12	5	42	10.6	8	39	17.0	0.60	0.189	1.912	0.5516	1.0000
C*14	2	45	4.3	0	47	0.0	5.22	0.244	111.718	0.4946	0.9999
C*15	3	44	6.4	4	43	8.5	0.76	0.177	3.267	1.0000	1.0000
C*16	2	45	4.3	2	45	4.3	1.00	0.165	6.053	1.0000	1.0000
C*17	1	46	2.1	1	46	2.1	1.00	0.100	9.968	1.0000	1.0000
C*01	2	45	4.3	2	45	4.3	1.00	0.165	6.053	1.0000	1.0000
DR17	12	35	25,5	12	34	26,1	0,97	0,390	2,422	1,0000	1,0000
DRB1*01	12	35	25,5	9	37	19,6	1,39	0,532	3,631	0,6212	1,0000
DRB1*04	9	38	19,1	11	35	23,9	0,76	0,288	2,015	0,6212	1,0000
DRB1*07	8	39	17,0	8	38	17,4	0,98	0,341	2,783	1,0000	1,0000
DRB1*08	2	45	4,3	1	45	2,2	1,67	0,211	13,150	1,0000	1,0000
DRB1*09	1	46	2,1	0	46	0,0	3,00	0,119	75,565	1,0000	1,0000
DRB1*10	1	46	2,1	1	45	2,2	0,98	0,098	9,758	1,0000	1,0000
DRB1*11	11	36	23,4	11	35	23,9	0,97	0,380	2,487	1,0000	1,0000
DRB1*12	7	40	14,9	2	44	4,3	3,30	0,740	14,675	0,1582	0,8934
DRB1*13	12	35	25,5	10	36	21,7	1,22	0,477	3,139	0,8080	1,0000

Table S2. (continued)

	Mothers High viral load[1]			Mothers Low viral load[4]			Odds ratio and 95% C.I.[5]			Fisher's Exact[6]	
Antigen	**Pos[2]**	**Neg[3]**	**%**	**pos**	**neg**	**%**	**OR[5]**	**lower**	**upper**	**P[6]**	**Pm[7]**
DRB1*14	2	45	4,3	6	40	13,0	0,34	0,075	1,564	0,1582	0,8934
DRB1*15	11	36	23,4	13	33	28,3	0,78	0,313	1,953	0,6411	1,0000
DRB1*16	1	46	2,1	2	44	4,3	0,57	0,073	4,530	0,6168	1,0000
DQ7	17	30	36,2	18	28	39,1	0,88	0,386	2,026	0,8322	1,0000
DQ8	8	39	17,0	8	38	17,4	0,98	0,341	2,783	1,0000	1,0000
DQ9	6	41	12,8	1	45	2,2	4,75	0,766	29,457	0,1109	0,5608
DQB1*02	15	32	31,9	18	28	39,1	0,74	0,317	1,705	0,5199	0,9941
DQB1*04	2	45	4,3	1	45	2,2	1,67	0,211	13,150	1,0000	1,0000
DQB1*05	17	30	36,2	16	30	34,8	1,06	0,458	2,455	1,0000	1,0000
DQB1*06	22	25	46,8	18	28	39,1	1,36	0,602	3,070	0,5317	0,9951

	Children High viral load[8]			Children Low viral load[9]			Odds ratio and 95% C.I.			Fisher's Exact	
Antigen	**pos**	**neg**	**%**	**pos**	**neg**	**%**	**OR**	**lower**	**upper**	**P**	**Pm**
A*01	16	31	34.0	15	32	31.9	1.10	0.470	2.567	1.0000	1.0000
A*02	24	23	51.1	23	24	48.9	1.09	0.488	2.420	1.0000	1.0000
A*03	10	37	21.3	13	34	27.7	0.72	0.282	1.813	0.6320	1.0000
A*11	4	43	8.5	6	41	12.8	0.66	0.185	2.364	0.7398	1.0000
A*23	4	43	8.5	0	47	0.0	9.83	0.514	187.879	0.1170	0.8248
A*24	10	37	21.3	9	38	19.1	1.14	0.423	3.042	1.0000	1.0000
A*25	1	46	2.1	1	46	2.1	1.00	0.100	9.968	1.0000	1.0000
A*26	1	46	2.1	2	45	4.3	0.59	0.074	4.630	1.0000	1.0000
A*29	2	45	4.3	0	47	0.0	5.22	0.244	111.718	0.4946	0.9999
A*30	0	47	0.0	2	45	4.3	0.19	0.009	4.100	0.4946	0.9999
A*31	3	44	6.4	5	42	10.6	0.61	0.149	2.476	0.7142	1.0000
A*32	2	45	4.3	6	41	12.8	0.35	0.077	1.601	0.2673	0.9871
A*66	1	46	2.1	1	46	2.1	1.00	0.100	9.968	1.0000	1.0000
A*68	5	42	10.6	3	44	6.4	1.65	0.404	6.703	0.7142	1.0000
B*07	9	38	19,1	11	35	23,9	0,76	0,288	2,015	0,6212	1,0000
B*08	12	35	25,5	10	36	21,7	1,22	0,477	3,139	0,8080	1,0000
B*13	2	45	4,3	1	45	2,2	1,67	0,211	13,150	1,0000	1,0000
B*18	2	45	4,3	4	42	8,7	0,52	0,105	2,574	0,4349	1,0000
B*27	3	44	6,4	6	40	13,0	0,49	0,125	1,924	0,3161	0,9998
B*35	6	41	12,8	7	39	15,2	0,83	0,265	2,572	0,7730	1,0000
B*37	1	46	2,1	1	45	2,2	0,98	0,098	9,758	1,0000	1,0000
B*38	2	45	4,3	2	44	4,3	0,98	0,161	5,923	1,0000	1,0000
B*39	1	46	2,1	2	44	4,3	0,57	0,073	4,530	0,6168	1,0000
B*44	16	31	34,0	8	38	17,4	2,37	0,916	6,148	0,0966	0,9034
B*47	0	47	0,0	1	45	2,2	0,32	0,013	8,043	0,4946	1,0000
B*49	1	46	2,1	1	45	2,2	0,98	0,098	9,758	1,0000	1,0000
B*51	**2**	**45**	**4,3**	**8**	**38**	**17,4**	**0,25**	**0,057**	**1,087**	**0,0502**	**0,6943**

Table s2. (continued)

	Children High viral load[8]			Children Low viral load[9]			Odds ratio and 95% C.I.			Fisher's Exact	
Antigen	**pos**	**neg**	**%**	**pos**	**neg**	**%**	**OR**	**lower**	**upper**	**P**	**Pm**
B*52	1	46	2,1	0	46	0,0	3,00	0,119	75,565	1,0000	1,0000
B*55	0	47	0,0	3	43	6,5	0,13	0,007	2,606	0,1170	0,9428
B*56	1	46	2,1	0	46	0,0	3,00	0,119	75,565	1,0000	1,0000
B*57	7	40	14,9	3	43	6,5	2,30	0,603	8,781	0,3161	0,9998
B*58	1	46	2,1	0	46	0,0	3,00	0,119	75,565	1,0000	1,0000
B60	10	37	21,3	7	39	15,2	1,48	0,522	4,164	0,5930	1,0000
B61	3	44	6,4	2	44	4,3	1,40	0,262	7,477	1,0000	1,0000
B62	6	41	12,8	10	36	21,7	0,54	0,186	1,595	0,2837	0,9995
B63	2	45	4,3	0	46	0,0	5,11	0,239	109,397	0,4946	1,0000
B65	1	46	2,1	1	45	2,2	0,98	0,098	9,758	1,0000	1,0000
C*01	2	45	4.3	2	45	4.3	1.00	0.165	6.053	1.0000	1.0000
C*02	6	41	12.8	10	37	21.3	0.56	0.191	1.636	0.4111	0.9983
C*03	18	29	38.3	17	30	36.2	1.09	0.478	2.498	1.0000	1.0000
C*04	10	37	21.3	9	38	19.1	1.14	0.423	3.042	1.0000	1.0000
C*05	5	42	10.6	10	37	21.3	0.46	0.151	1.418	0.2596	0.9729
C*06	10	37	21.3	6	41	12.8	1.79	0.611	5.229	0.4111	0.9983
C*07	25	22	53.2	26	21	55.3	0.92	0.412	2.053	1.0000	1.0000
C*08	1	46	2.1	1	46	2.1	1.00	0.100	9.968	1.0000	1.0000
C*12	7	40	14.9	2	45	4.3	3.37	0.758	14.995	0.1582	0.8733
C*14	1	46	2.1	0	47	0.0	3.07	0.122	77.169	1.0000	1.0000
C*15	1	46	2.1	3	44	6.4	0.41	0.058	2.901	0.6168	1.0000
C*16	1	46	2.1	0	47	0.0	3.07	0.122	77.169	1.0000	1.0000
C*01	2	45	4.3	2	45	4.3	1.00	0.165	6.053	1.0000	1.0000
C*02	6	41	12.8	10	37	21.3	0.56	0.191	1.636	0.4111	0.9983
DR17	11	36	23,4	11	35	23,9	0,97	0,380	2,487	1,0000	1,0000
DRB1*01	14	33	29,8	8	38	17,4	1,96	0,747	5,146	0,2228	0,9514
DRB1*04	**5**	**42**	**10,6**	**17**	**29**	**37,0**	**0,22**	**0,075**	**0,634**	**0,0034**	**0,0401***
DRB1*07	15	32	31,9	7	39	15,2	2,51	0,935	6,744	0,0868	0,6636
DRB1*08	2	45	4,3	1	45	2,2	1,67	0,211	13,150	1,0000	1,0000
DRB1*09	0	47	0,0	1	45	2,2	0,32	0,013	8,043	0,4946	0,9997
DRB1*10	1	46	2,1	1	45	2,2	0,98	0,098	9,758	1,0000	1,0000
DRB1*11	5	42	10,6	5	41	10,9	0,98	0,278	3,432	1,0000	1,0000
DRB1*12	6	41	12,8	3	43	6,5	1,95	0,496	7,640	0,4856	0,9997
DRB1*13	16	31	34,0	11	35	23,9	1,62	0,662	3,948	0,3621	0,9955
DRB1*14	3	44	6,4	5	41	10,9	0,59	0,146	2,419	0,4856	0,9997
DRB1*15	10	37	21,3	14	32	30,4	0,63	0,249	1,579	0,3506	0,9944
DQ7	14	33	29,8	12	34	26,1	1,20	0,489	2,919	0,8180	1,0000
DQ8	**5**	**42**	**10,6**	**14**	**32**	**30,4**	**0,29**	**0,098**	**0,856**	**0,0218**	**0,1428**
DQ9	7	40	14,9	2	44	4,3	3,30	0,740	14,675	0,1582	0,7004
DQB1*02	19	28	40,4	17	29	37,0	1,15	0,505	2,633	0,8322	1,0000
DQB1*04	2	45	4,3	1	45	2,2	1,67	0,211	13,150	1,0000	1,0000

Table s2. (continued)

	Children High viral load[8]			Children Low viral load[9]			Odds ratio and 95% C.I.			Fisher's Exact	
Antigen	**pos**	**neg**	**%**	**pos**	**neg**	**%**	**OR**	**lower**	**upper**	**P**	**Pm**
DQB1*05	19	28	40,4	13	33	28,3	1,70	0,722	3,992	0,2764	0,8961
DQB1*06	22	25	46,8	21	25	45,7	1,05	0,467	2,345	1,0000	1,0000

[1] HLA alleles frequencies in the mothers with children with viral load measured on DBS above the median (3.2 log (IU/ml)); [2] Mothers positive for the specific HLA allele; [3] Mothers negative for the specific HLA allele; [4] HLA alleles frequencies in the mothers with children with viral load measured on DBS below the median; [5] Odds ratio (OR) and 95% confidence intervals (95% C.I.) calculated based on the Woolf-Haldane method; [6] Two-sided Fisher's exact test comparing HLA alleles frequencies between high and low viral load group; [7] Pm: corrected p-values for multiple comparisons (Šidák method); [8] HLA alleles frequencies in the children with viral load measured on DBS above the median; [9] HLA alleles frequencies in the children with viral load measured on DBS below the median. * Significant p-value after correction.

CHAPTER 7

GENERAL DISCUSSION

This thesis aimed to identify prognostic markers, for short- and long-term clinical outcome, and correlates of protection, for future vaccine development. In order to identify such biomarkers, a retrospective nationwide cohort of children with (n=125) and without congenital CMV infection (cCMV) (n=263) was used. The findings of this thesis allowed us to get more insights into cCMV pathogenesis, and into the potential processes leading to immune dysfunction, and therefore to a worse clinical outcome. Thus, Chapter 7 delves into some important aspects of each chapter first, with particular attention to the possible mechanisms of cCMV pathogenesis and its clinical implications, i.e *Summarising Discussion*. This is followed by a paragraph that attempts to integrate all the findings in one final model, with special emphasis on what could be done in the future research to complete the findings of this thesis, i.e. *Concluding Discussion*.

7.1. SUMMARIZING DISCUSSION

7.1.1. Neonatal T and B cell markers in relation to symptoms, LTI and viral load

In **Chapter 2** the immune system of the neonates was assessed by means of quantification of the most common DNA rearrangements occurring at the receptor level of B cells, αβ and γδ T cells. Our findings suggested that the intrauterine infection may have an effect on thymopoiesis, leading to a reduced thymic production of αβ T cells. Because CMV can infect thymic ephitelial cells (1, 2), and because smaller thymuses have been described in CMV infected newborns (3), our hypothesis seems plausible. Infected thymic ephitelial cells, which are fundamental for T cell development and maturation, could impair the output of naïve T cells. T cells play a central role in controlling CMV infection and disease, therefore, if the initial impairment was permanent one could assume a reduced long-term control of the infection, with a consequent worse long-term outcome. However, the reduced thymic output at birth was not associated to long-term impairments (LTI), suggesting that it is temporary and that other mechanisms are in place to compensate such initial impairment. In our cohort, the infected group had higher number of γδ T cells. The role of γδ T cells during cCMV has already been shown, but in the context of primary maternal infection, with antiviral activity when incubated with CMV-infected cells (4). Therefore, they could be potential candidates for such compensation, at least in the early phase of the infection, because they develop earlier than αβ T cells during the immune ontogeny, and react rapidly upon activation (5). Having higher levels of γδ T cells at birth did not correlate with a better long-term outcome, but positively correlated with CMV viral load, further suggesting their role in the early phase of cCMV. Interestingly, the reduced thymic output did not get worse with higher viral load. However, we do not know if higher blood viral load corresponds to higher viral load in the thymus. Therefore, we cannot exclude that a more severe local infection leads to a more reduced thymic output. The infected group had an increased number of circulating and newly derived bone marrow B cells compared to the control group. Therefore, even though we know that CMV can infect the bone marrow, a different pathogenetic mechanism may be assumed between the αβ T cell and B cell compartment, because, overall, cCMV does not induce a reduced B output as for αβ T cells. As the number of circulating and newly derived bone marrow B cells were comparable in all analyses, we concluded that cCMV leads to an increased B cells production in the fetal period, rather than to an extensive proliferation.

And such a B cell production positively correlated with viral load, suggesting a causal relationship between high viral loads and cell activation, as previously suggested (6). Although, overall, infected neonates had higher number of B cells at birth, for those who developed LTI, this number was lower, comparable to non-infected controls. It is tempting to speculate that this difference in numbers reflects a difference in e.g. the capacity to generate long-lived plasma cells, memory B cells, Abs or support effector functions of immune cells. What is the trigger to this dysfunction is difficult to determine in our cohort, as it seems that viral load is not related to LTI development. Finally, none of the immunological markers included in this study was associated with symptoms at birth.

7.1.2. Neonatal metabolic markers in relation to symptoms, LTI and viral load

In **Chapter 3** the metabolism of neonates was assessed by means of quantification of the metabolites extracted from dried blood spots (DBS) for the screening of the rare genetic metabolic disorders (essential amino acids, hormones, carnitines and enzymes). During infections, there is a general increase of metabolic requirements. The virus is a considerable burden on cells because they are dependent on the host's energy and biosynthetic pathways to replicate (7). For example, CMV envelope is enriched for longer chain fatty acids, and its infectivity is reduced by the inhibition of the host enzyme involved in their biosynthesis. This is why we found a positive correlation between palmytoilcarnitine, which reflects the level of palmitate (precursor of longer chain fatty acids) and viral load. Additionally, the immune system increases the proteins biosynthesis to allow a broad-spectrum of anti-microbial functions (7). In our cohort, cCMV influenced the metabolites only in premature neonates. If cCMV occurs in premature neonates, they may not be able to cope, or at least not as good as term neonates, with the general increased energetic and biosynthetic demand during an infection, with a consequent lower level of essential aminoacids reflected in whole blood. However, since a certain degree of correlation has been shown between cCMV and prematurity (8), we cannot establish which was first, and most likely it is a combination of factors. Although we did not find a significant difference in the number of premature between cCMV+ and cCMV-, we found more prematurity in the infected group that developed LTI than in the infected group that did not. Interestingly, prematurity, both early, moderate and late, has been shown to be associated with LTI similar to those reported for cCMV (9). Obviously, prematurity, even if not caused by cCMV, will not help to fight the effect of a CMV infection.

Finally, in past pediatric cohorts more female were reported with neurological impairments and cerebral ultrasounds findings, even though the number of infected males was higher than the number of females (10). This bias was explained by assuming that the most severely affected fetuses died in utero and that the damage in the survivers was caused by the immune inflammatory response to CMV and by direct cytopathic effect (CPE) (10). Since in our cohort neonates were not selected based on their abnormal ultrasonographic state, or on the maternal primary infection, we did not enrich for severe cases. And this may be the reason why we did not detect differences in male/female proportion between symptomatic and asymptomatic. However, we found a higher proportion of infected males that developed LTI, whereas this was not observed in the control group (unpublished observations). The same differences that lead to higher resistance to bacterial and viral

infections in female, or higher susceptibility to autoimmune diseases when an inadequately high immune response occurs, might be involved in the long-term response to CMV as well. The overall morbidity rate is higher in males than females (11) and, in absence of immunopathology, the sex-related differences might lead to a more effective long-term CMV control in female. Interestingly, infections as measles, mumps and RSV, other relevant problems in neonates, can cause more extensive complications in male (12). Such differences certainly merit more research because sex-specific data on long-term CMV response in congenitally infected newborns are missing.

7.1.3. Neonatal transcriptome in relation to LTI and viral load

In **Chapter 4** neonatal trascriptome was assessed my means of sequencing RNA extracted from DBS. Overall, the differences in whole blood gene expression profile were too small to be able to identify prognostic markers. This happened for a number of reasons extensively discussed in the paper. However, since gene expression analysis captures a snapshot of the cellular activity, considered to be the result of the response to genetic, environmental and epigenetic factors (13), we could make important observations on cCMV immuneregulation. Indeed, DBS mainly reflect the neonatal immune system because they are produced by spotting whole blood on filter paper. Three main observations characterized this study. First of all, high CMV viral load is the main initiator of the transcriptional differences observed in whole blood. Consequently, the differences between CMV+ and CMV- are diluted out because of the presence of low viral load individuals in the CMV+ group. Second of all, numerous antiviral and NK cells activation genes were positively associated with CMV viral load, suggesting the involvement of the innate immune system in response to higher CMV viral load in the fetus. This has been shown before in human first-trimester maternal-decidual tissues. Here, CMV infection upregulated genes related to antiviral innate immune response pathways, with particular emphasis on immune cell activation, proliferation, and trafficking patways (14). Therefore, what occurs at the maternal-fetal interface may be reflected at birth. Third of all, anti-inflammatory markers, such as the cytokine IL-4, were associated with congenitally infected children that did not develop LTI, suggesting that LTI pathogenesis may be partially attributable to an uncontrolled inflammation. The molecular mechanisms of LTI development are largely unknown. In neonates with neurological impairments and cerebral ultrasounds findings at birth, an uncontrolled inflammation together with a direct CPE are believed to be the responsible (10). Whereas, the late-onset hearing loss is believed to be the result of a chronic productive infection that occurs throughout childhood (15, 16). This suggests that the long-term immune response against cCMV is dysfunctional. Therefore, it cannot be excluded that such a dysfunctional immune response can lead to an uncontrolled inflammation that additionally contributes to tissue damage. Similarly to IL-10, IL-4 has been shown to possess similar capacity of down-regulating the production of pro-inflammatory mediators by microglia, both in humans and in mice (17-19), and its neuroprotective effect is believed to be based on the inhibition of brain inflammation (20). Additionally, in a cohort of healthy CMV infected individuals, the CD4 T-cell response associated with a protective immunity included the production of IL-4 (21). Considering that half of the neonates (in this chapter) developing LTI had microcephaly at birth, it is tempting to speculate that anti-

inflammatory markers may help in limiting the long-term tissue damage. Finally, what causes this immune long-term immune dysfunction is unknown, and T cell exhaustion could be one potential mechanimsm. However, in our cohort, T cell exhaustion that characterized the infected group, and was more marked with high CMV viral load, did not seem to play a role in the development of LTI, further supporting the reversibility of such phenomenon.

7.1.4. Maternal-child HLA, expressed at the placenta, in realtion to symptoms, LTI and viral load

In **Chapter 5** the maternal and fetal HLA background was assessed by means of HLA-C, HLA-E and HLA-G typing in both mothers and neonates, and the mothers were additionally typed for KIRs. We wanted to evaluate whether the maternal-child combination of HLAs expressed at the placenta, as well as the individual HLAs, may influence cCMV outcome. cCMV pathogenesis, and consequently its clinical outcome, is the result of a complex interplay between viral, maternal, placental, fetal and child factors. Within each compartment there are many aspects to be taken contemporarily into consideration, and given the complexity of the placental immune cross-talk, a short introduction is preparatory to this section's discussion.

Maternal-fetal imme cross-talk summary: the mother has a complicated task, she has to guarantee the immune tolerance to the fetus, by definition a semi allograft, and at the same time has to protect the fetus from infections. These two tasks involve opposite mechanisms of the same cellular populations, e.g. CD8+ T cells. Therefore, a proper compromise should be established. T cells represent the 5-20% of total decidual lymphocytes in the early phases of gestation, whereas this percentage becomes 40-80% at the end of pregnancy (22, 23). The majority of CD8+ T cells are activated effector memory T cells with naïve T cells almost absent (24-26), therefore cells that are highly Ags experienced/differentiated (27). Maternal fetus-specific T cells, i.e. specific for paternal Ags such as HLA-A, HLA-B and HLA-C, have been shown both in the maternal peripheral blood, and at the maternal-fetal interface (28, 29). The frequency of B cells in the decidua is very low (30), and this allows a certain degree of protection from Abs-mediated effects (31, 32). Monocyte are recruited to the decidua through interaction with the trophoblast-derived chemokine receptor ligands, they can differentiate into DCs (33), and are approximately <1% of total cells in the first trimester (23). Finally, decidual natural killer cells (dNK) cells are the most abundant leucocyte population, at least during the first trimester of pregnancy (34), and can also be generated locally (27). NK cytotoxicity is controlled by a combination of both activating and inhibitory receptors (35-38). In normal conditions, several mechanisms are in place to prevent a detrimental maternal immune response against the fetus. However, endogenous and exogenous factors can alter such delicate balance. An endogenous factor may be a not so favorable maternal KIR/fetal HLA-C combination (27), that skews dNK response towards an inhibitory response, which in turn negatively affects trophoblast invasion (39). An exogenous factor may be an infection, such as cCMV, which triggers an inflammatory response. This alters normal trophoblast invasion, induces apoptosis and placental dysfunction, with a consequent fetal damage, e.g. IUGR (40-42).

Our findings added to this picture the following: if cCMV occurs at the backdrop of certain maternal-fetal HLA combinations such inflammation may be worsened, with a consequent worse

clinical outcome at birth. In particular, a reduced maternal control over cCMV, due to HLA-G del/del genotype which leads to higher protein, may induce an increased viral and cellular burden at the placenta. Here, a fetal allo-Ags recognition by the maternal immune system may contribute to placental inflammation and dysfunction through direct damage. This was suggested by the increased percentage of HLA-E mismatches (mm) and HLA-C mm in the group of symptomatic neonates. Since in normal healthy pregnancies a shift towards more placental activated T cells in the presence of HLA-C mm has been shown (29), our theory seems plausible. Additionally, considering that HLA-E and HLA-C can present CMV Ags, a suboptimal viral clearance seems reasonable because maternal cells would not efficiently recognize CMV presented in the context of the allo-HLA by fetal cells.. This is further supported by the higher percentage of mm in the high viral load group. Finally, the absence of maternal HLA-C belonging to the C2 group was associated with symptoms at birth, most likely because this skews NK cell activation towards a pro-inflammatory state as the HLA-C1 has less inhibitory capacity on dNK cells. Indeed, dNK cells do not interact only with fetal ligand, but also with maternal ligands as part of the internal immune homeostasis. Taken together, these factors may contribute to placental inflammation, dysfunction and a worse outcome at birth. Finally, prematurity has been associated to chronic placental inflammation in the absence of infections (43), and given the relatively high percentage of prematures in our cohort, the possibility of an inflammatory effect independent of CMV needed to be considered as well. Excluding the premature from the analysis, the results showed slight changes of p-values, but not of trends in the percentages. Removing 11 individuals from the symptomatic group led to lack of statistical power as we are left with only 8 individuals, and since we observed the same trends of percentages we could conclude that the associations we found were not influenced by prematurity. Although this is logical, from a statistical point of view we cannot fully exclude it. However, taking into account the association between cCMV and prematurity (8), it is plausible to assume that prematurity is an effect of the combination of cCMV and aforementioned HLAs.

Finally, as previously mentioned, LTI development can be considered the result of a long-term immune dysfunction that leads to an uncontrolled viral replication and inflammation. Our findings added to this picture the following: HLA-C non-inherited maternal antigens (NIMAs) may support a long-term uncontrolled viral replication by means of the tolerance induced in the fetus towards NIMAs, which indirectly induce a tolerance to CMV. The NIMA effect has been shown in transplantation because it improves the outcome. Despite the exact molecular mechanism is largely unknown, several observations led to an hypothetical mechanism, which will be described in the paragraph below. Additionaly, as previously mentioned, prematurity has been shown to be associated with LTI similar to those reported for cCMV (9). Given the relatively high percentage of premature in our cohort, we considered the possibility of an effect independent of CMV. In our cohort, the results did not change, suggesting that prematurity does not influence the association we found between HLA-C NIMAs and LTI. By removing 6 individuals from the group with LTI, and 5 in the group without, we are left with 21 vs 64 which does not lead to small numbers, as observed for symptoms at birth.

Hypothetical mechanism of the NIMA effect in cCMV: in order for this effect to occur, the fetal immune system has to "see" the maternal cells carrying NIMA. This is due to a mutual exchange of

cells between mother and fetus that starts during pregnancy and continues with breast feeding, leading to microchimerism (44, 45). These allogeneic cells seed in different organs in both mothers and children. In the latter, they can reach several fetal tissues such as bone marrow, blood, spleen, heart, lungs, pancreas and lymph nodes (46-49). The NIMA "recognition" can occur through different mechanisms, direct and indirect pathways. Fetal T cells can recognize NIMAs directly as intact antigens expressed on maternal cells or indirectly as peptides presented by the shared HLA. Fetal T cells can recognize NIMA derived peptides presented by fetal antigen-presenting cell (APC) in HLA Class I and Class II, and fetal APC can "meet" maternal cells in many fetal tissues. However, this recognition will not lead to immune activation but to immunomodulation of both T- and B-cells. When fetal CD4 T cells, that have already been modulated by NIMA, interact with APC's expressing both NIMA and peptides derived from CMV, this will lead to downregulation of the immune response to CMV (linked immune suppression) (50, 51).

7.1.5. Maternal-child HLA, not expressed at the placenta, in realtion to symptoms, LTI and viral load

In **Chapter 6** the maternal and fetal HLA background was assessed by means of HLA-A, HLA-B, HLA-DR and HLA-DQ typing in both mothers and neonates. We wanted to evaluate whether the maternal-child combination of HLAs not expressed at the placenta, as well as the individual HLAs, may influence cCMV outcome. We decided to study the HLAs that are not expressed at the placenta because an allogeneic response can still occur (28, 52-54). Viral infections can increase the levels of pro-inflammatory cytokines and chemokines at the maternal-fetal interface, and intensify the decidual T cell influx (22, 55). Therefore, the increased level of allogeneic maternal cells specific for fetal Ag that are not expressed by the trophoblast, may contribute to placental immunopathology by increasing inflammation. This hypothesis seemed plausible because in Chapter 5 placental immunopathology seemed to be responsible for placental dysfunction, and a worse outcome at birth. However, mm and NIMAs for those HLAs not expressed at the placenta were not associated with a worse short- and long-term outcome in our cohort. However, it is worth to mention the trend of higher HLA-A and HLA-B mm percentages in the symptomatic group, which therefore are considered to have a small effect in the context of cCMV. Furthermore, HLA-C NIMA can have a more important role compared to NIMA of other HLAs. CMV has developed strategies to evade host immunity, and to establish latency by down-regulating classical HLA molecules and up-regulating non-classical HLA, while maintaining HLA-C expression in order to avoid NK cells activation. The important role of HLA-C during CMV infection is further supported by the fact that certain HLA-C-restricted CD8 T cells have more efficient antiviral functions than HLA-A or HLA-B (56). Thus, if an HLA molecules is not expressed, the NIMA Ag cannot be processed, and the NIMA-specific regulatory mechanisms do not occur as efficiently as if the Ag is expressed. Additionally, the degree of the NIMA effect may also vary according to the percentage of microchimeric cells that engrafted fetal tissues. In fetal lymph nodes a predominance of hematopoietic cells was observed, with a frequency between 0.0035% to 0.83% (49), and others have reported similar % for non-lymphoid organs both from fetuses, neonates and adults (46, 57).

In this chapter, we additionally evaluated the individual, maternal and child, HLAs in relation to neonatal CMV viral load. The main finding is that HLA-DRB1*04 seemed to be protective, or at least to better control the infection, as its frequency was increased in the neonates with lower viral load. Such an effect did not lie in the maternal-fetal combination, but in the presence of these Ags in the child. HLA Class II is involved in the support of CTL and humoral response, which are fundamental players in controlling CMV infection. This suggests that a favourable CMV-HLA II combination can have an important dual effect.

7.2. CONCLUDING DISCUSSION

Several approaches have been used to explore prognostic markers for long-term outcome, but not all of them seemed promising, although all of them were useful to get more insights into cCMV pathogenesis. An important aspect should be kept in mind, this is an associative study, and, as such, it needs confirmation, both in other associative studies and experimentally. The first would allow us to determine whether the findings of this thesis, mainly those genetic, can be reproduced in other comparable cohorts. The second would allow the determination of the molecular mechanisms, which is fundamental to design any innovative intervention. The neonatal immune markers, through DNA quantification of the most common TCR and BCR rearrangements from DBS, together with the maternal-child HLA background, through typing DNA from buccal swabs, seemed to be quite promising for prognostic markers, and certainly merit further evaluation. Whereas the small effect of cCMV on the transcriptome profile from DBS should first be confirmed with fresh material before excluding such an approach. Finally, the neonatal metabolism, which was assessed when the material was fresh, did not look like an encouraging approach for finding prognostic markers in the context of cCMV. In the following paragraphs, an attempt to integrate all the findings in one final model is presented in relation to cCMV, symptoms at birth and LTI development (Fig. 1). A final paragraph on the role of CMV viral load is included because it was a recurrent topic in all chapters.

7.2.1. Congenital CMV infection

cCMV affected the neonatal immune system by reducing the thymic output of αβ T cells, increasing circulating γδ T cells as well as newly formed and circulating B cells (Chapter 2). cCMV did not induce extensive proliferation of B cells, rather increased the general production (Chapter 2), and induced exhaustion of T cells (Chapter 4). CMV viral load was an important determinant for shaping the immune system not only in relation to the number of circulating γδ T cells, newly formed and circulating B cells, but also in relation to the whole blood transcriptome of innate immune system and NK cell activation (Chapter 4). This raises a central point, i.e. the presence of low viral load neonates in the CMV+ group may mask important effects in the immune system. The only immune marker not associated with viral load was the thymic output of αβ T cells, however, we cannot conclude that the viral load would not influence this compartment because we do not have a marker for circulating αβ T cells. And we do not have a marker for the number of newly formed γδ T cells in order to see whether, similarly to αβ T cells, cCMV induces a reduced output. Therefore, in future research it would be important to include these two markers to have the complete picture. The innate immune

system, NK cell activation and γδ T cells may be important mechanisms to control CMV in the early phase of infection, where the output of αβ T cells is reduced, and the CMV-specific CTL and humoral responses are still developing. As both the CTL and the humoral responses are fundamental for controlling CMV infection, a favorable HLA Class II-CMV peptide complex would support more efficiently both arms of the adaptive immune system with a consequent more efficient viral control (Chapter 6). Therefore, in future studies, it would be important to determine which CMV peptide is presented in the context of HLA-DRB1*04 as this could be exploited in future vaccine development. Moreover, although both the virus and the immune system activation impose an important burden on the cellular metabolism, as it has been shown *in vitro*, overall the effect was not strong enough to be reflected in whole blood in the general population but only in premature neonates (Chapter 3). Premature neonates may not be able to efficiently cope with the general increased metabolic requirements, with a consequent lower level of essential aminoacids, fundamental for the correct development. Therefore, future studies are needed to evaluate the clinical implications of cCMV in this special category of neonates. Finally, similarly to the neonatal immune system, CMV viral load appeared to be an important determinant in shaping the neonatal metabolism. Indeed, C16, which reflects the level of palmitate, i.e. precursor of longer chain fatty acids for which CMV is enriched for, was positively correlated to CMV viral load.

7.2.2. Symptoms at birth

The number of various immune cells was not associated with a worse outcome at birth (Chapter 2). One plausible explanation may be the fact that the clinical signs included in the definition of symptoms at birth in our cohort are rather general. Indeed, the mechanisms that lead to neonatal hepatosplenomegaly, petechiae, purpura, thrombocytopenia, neutropenia or elevated ALAT may not be due entirely to a dysfunction of the neonatal immune system per se. Whereas in those symptoms that are more specific of cCMV, such as microcephaly, one could assume that the neonatal immune system may have a more important role. E.g., in solid organ-transplanted patients, γδ T cell expansion was associated with resolution of CMV infection and less symptomatic CMV disease, and late γδ T cells expansion was correlated with a more intense and durable CMV infection (58). This can potentially be also the case in our cohort if only we could assess all immunological markers in relation to the individual clinical signs. In our cohort1, symptoms at birth seemed to be mainly caused by a placental dysfunction as a result of a multifactorial process that starts in the mother and continues in the placenta (Chapter 5). This seems plausible because a placental dysfunction can lead to general fetal harm. The genetic factors involved are: maternal HLA-G del/del genotype that increases the cellular and viral burden at the placenta; HLA-E mm and HLA-C mm that induce a fetal allo-Ags recognition by the maternal immune system, and a suboptimal viral clereance; absence of maternal HLA-C belonging to the C2 group that skews dNK cells activation towards a pro-inflammatory state (Chapter 5). Here, mm that are specific for HLA not expressed at the placenta, such as HLA-A mm and HLA-B mm, may partially contribute to placental inflammation (Chapter 6). Evaluating the molecular mechanisms behind the influence of such genetic factors during cCMV in relation to symptoms at birth would be crucial for creating any interventions. This is not easy in

a prospective study, but to start with maternal blood samples would be useful to compare the CMV-specific immune system in relation to HLA-G genotype. Additionally, placental specimens would be necessary to evaluate the effect of the maternal HLA-G and HLA-C genotypes, as well as maternal-fetal HLA-C mm and HLA-E mm, in the cellular and structural composition of the placenta. And the CMV viral load should always be taken into consideration.

7.2.3. Long-term impairments

In our cohort, a dysfunctional immune response that leads to an uncontrolled viral replication, combined with an uncontrolled inflammatory response, appeared to be responsible for LTI development. This most likely involves both the effector and regulatory mechanims. Neonates developing LTI had lower numbers of newly formed and circulating B cells at birth, suggesting some sort of long-term dysfunction (e.g. in the capacity to generate long-lived plasma cells, memory B cells, antibodies (Abs) or support effector functions of immune cells), or reduced capacity to control the infection (Chapter 2). As both CTL and humoral response are necessary to control CMV infection, it would be extremely interesting in future research to see whether infected neonates that developed LTI had lower number of circulating αβ T cells at birth, both total and CMV-specific. What is the trigger of this difference in B cells number between infected neonates that develop LTI and those who do not is impossible to establish in our cohort. Importantly, the exhaustion that we described for T cells (Chapter 6), but that has also been described for B cells in the context of other chronic infections such as HIV (59), did not seem to be so permanent to cause a long-term dysfunction. The exhaustion temporariness is not surprising because in a murine model of chronic infection, the blockade of PD-1 resulted in reversion of the exhaustion (60). A genetic factor may contribute to a long-term dysfunction. In our cohort, HLA-C NIMA appeared to be partially responsible for LTI development (Chapter 5). The so-called NIMA effect, i.e. immune tolerance to NIMA, indirectly can induce a tolerance to the infection with a consequent reduced viral control. This effect, that can influence both T and B cells, is due to microchimeric cells that persists at least till early adulthood (49, 61). Demonstrating the role of a genetic factor for LTI development is more difficult because of the late onset of certain LTI, and of different pathogenetic mechanisms that may be responsible for different impairments such as hearing loss and motor impairment. One way to start would be to compare the neonatal CMV-specific immune response with and without HLA-C NIMA, and repeat this over time, in relation as well to CMV viral load. The immune dysfunction that leads to an uncontrolled viral replication may be followed by dysfunctional compensatory mechanisms that in turn lead to an uncontrolled inflammatory response. Indeed, in our cohort, anti-inflammatory markers characterized infected neonates that did not develop LTI, further supporting the role of inflammation as additional determinant for tissue damage (Chapter 4). In the context of development of LTI, the sex-related differences in the immune system should be taken into consideration as well (Chapter 3). Finally, prematurity has been shown to be associated with LTI similar to those reported for cCMV (9), as well as to chronic placental inflammation also in the absence of infections (43). However we believe that it is not the prematurity per se that is responsible for the LTI in our cohort. First of all, although prematurity is not always used as a criterion

in the definition of "symptoms at birth", it has been shown that cCMV is frequently accompanied by prematurity (8). When focusing on symptomatic groups (with prematurity not used as a criterion) the percentage of prematurity is usually around 36% (62). The percentage of symptomatic children in the infected group in our cohort, including those that were premature, was 18%, which is similar to that found in previous studies. The percentage of prematures in the infected group (10.8%) was similar to that found by others (and only slightly higher than in the general population), although others have found lower percentages (8, 63). Despite the fact that the percentage of premature neonates were relatively high in our cohort, our data showed that the association we found between HLAs and cCMV clinical outcome is not confounded by prematurity. However, there is no doubt that prematurity will not be beneficial for the undisturbed child development, but cCMV and HLAs may have a synergistic effect on prematurity, and these three factors may all contribute to LTI development.

7.2.4. CMV viral load and DBS testing

CMV viral load in our cohort was not significantly associated to symptoms at birth nor to LTI. The role of CMV viral load in the cCMV clinical outcome still needs to be elucidated as some previous studies have related the viral load to a worse outcome (62, 64), whereas others have not (65-67). However, there is no standardized way of defining symptoms at birth or LTI, and the CMV viral load in blood of infected neonates may vary depending on the timing of infection, whether there was a maternal primary or secondary infection, or on the specific compartment impaired. Indeed, it is tempting to speculate that between a neonate with cCMV that has hearing loss at birth and a neonate with cCMV that has elevated liver transaminases, the peripheral CMV viral load differs. Unfortunately, due to lack of statistical power we could not evaluate how whole blood CMV viral load changes in relation to the different compartments impaired. For all aforementioned reasons, if any role of viral load in blood exists, it may have been diluted in our cohort. In future research, defining the role of viral load in relation to individual clinical outcomes, standardizing the definition of symptomatic disease and LTI development is absolutely essential in order to be able to draw any conclusion on pathogenesis. Indeed, what we concluded in this thesis can very well not be the case in another cohort just because different definitions are used. Nevertheless, this cohort study, retrieved from a large population screening, does reflect a real population of newborns with cCMV in all its diversity, ranging from no symptoms at birth and no LTI to symptoms at birth with severe LTI. This would be the situation we will have to deal with in a universal screening setting. Additionally, DBS testing for cCMV diagnosis may be seen as a potential limitation. Indeed, the golden standard method for cCMV diagnosis is the urine testing because of the high viruria observed in congenitally infected infants (65), but saliva is considered equally reliable (68), and CMV viral load is usually lower in blood than in urine (65). DBS can be considered a proper and reliable alternative specimen to blood, because a positive correlation between CMV viral load measured on DBS and CMV viral load measured on whole blood/ plasma, both in the context of cCMV and CMV in transplantation, was shown (69, 70). These studies suggested that DBS may be used not only for diagnosis but also for monitoring response to therapy. Moreover, DBS testing has a reputation of lower sensitivity, and the DNA/RNA content is believed to be more prone to degradation. Importantly, even considering

the relative reduction of CMV viral load on DBS in time (69), the specimens that could be potentially more affected from the aforementioned differences between blood and urine/saliva are the ones with low viral load. This implies that low-positive neonates may be found in the CMV negative group. However, with the high sensitivity of our PCR (estimated > 85%), high specificity (> 99.9%) and the cCMV birth prevalence of 0.5%, the chance of a CMV false-negative result is 1/1000 (71). Therefore, the influence of the sensitivity of the CMV PCR on DBS on the conclusions in all chapters can be considered negligible. Despite the aforementioned potential limitations, the use of DBS, that are normally collected at birth for the screening of rare genetic metabolic disorders, has several advantages over other specimens. Since the DBS are routinely collected, they are available almost worldwide, and allow large-scale retrospective studies for long-term cCMV outcome, which would be challenging in a prospective setting. Additionally, CMV detection on DBS does not require special facilities, is not expensive and an automation can be performed in order to include many specimens at the same time further reducing the price. However, efforts in improving these aspects and the standardization of such a method are necessary for the introduction in the screening program.

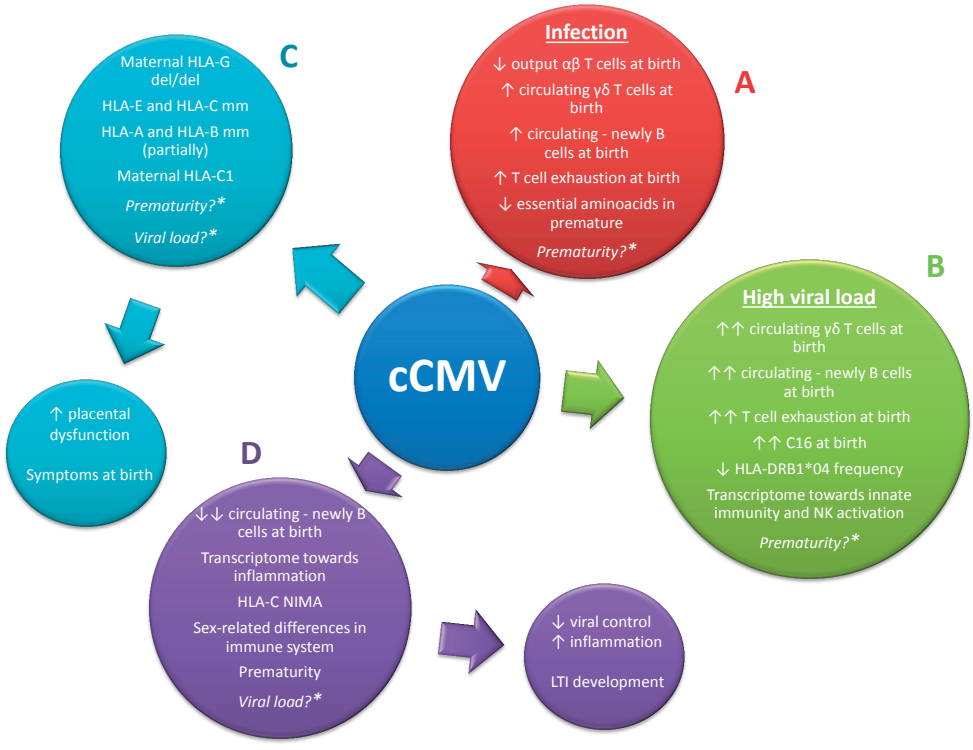

Figure 1. Final model of cCMV pathogenesis. A) Overall effects of cCMV in relation to the neonatal immune system and metabolism. B) Effects of higher CMV viral loads in relation to the neonatal immune system and HLA Class II. C) Genetic factors that in presence of a cCMV may lead to placental dysfunction, and consequently to symptoms at birth. D) Genetic and immune factors that in presence of a cCMV may lead to reduced viral control and increased inflammation throughout childhood, and consequently to LTI development.
* Indicates factors whose role in the pathogenesis of cCMV and cCMV-related disease need further evaluation.

To conclude, any future intervention should focus on supporting both arms of the adaptive immune response in order to efficiently control the chronic viral replication, with particular attention to regulating the inflammatory response. This seems achievable with a vaccine, though the target population may vary depending on the goal of such vaccine. However, as the way to a licensed vaccine seems to be long, the *tertiary prevention* sounds like a good compromise. By means of *tertiary prevention* we would like to prevent, or at least positively affect, the short- and/or long-term impairments. How can we efficiently intervene to limit a progressive permanent damage that has a late onset manifestable in years after birth? We have to understand the pathogenesis of cCMV, and to identify a biomarker that differentiate these groups. The majority of congenitally infected neonates are asymptomatic at birth and have a good prognosis for a normal long-term development, therefore the comparison of their immunological condition with that of the symptomatic infected neonates with a worse long-term outcome, as well as those asymptomatic that develop the same LTI, should be the focus in future research. Importantly, the approaches used would need to be powerful enough to get subtle differences that are likely to be expected between asymptomatic that develop LTI and asymptomatic that do not. In parallel with this, it would be essential to determine whether treatments offered to symptomatic neonates, ranging from antivirals to audiological and motor follow-up, are beneficial in those asymptomatic neonates that develop LTI. For example, whether (val)ganciclovir treatment of asymptomatic neonates with higher probability to develop hearing problems actually has a positive effect on the hearing compared to a similar group that did not get such treatment. Maybe the side effects of such treatment would exceed the positive effect in a way that would be better to somehow boost their immune system. However, an even earlier goal should be the standardization of the definitions of symptomatic disease and LTI development, as well as the role of viral load in the clinical outcome, without which no reliable conclusion on the actual cCMV pathogenesis, and its clinical consequences, can be drawn. Concluding, understanding the pathogenesis of cCMV would allow to figure out why certain children develop LTI and others do not. In turn, this would provide the necessary biomarkers to predict outcome and stratify patients according to the risk. On one hand we could define who benefits most from certain clinical interventions; and on the other hand we could define correlates of protection to be used in future vaccine trials.

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APPENDIX &

ABBREVIATIONS

ENGLISH SUMMARY

SAMENVATTING

ITALIAN SUMMARY

LIST OF PUBLICATIONS

ACKNOWLEDGEMENTS

CV

ABBREVIATIONS

CMV	Cytomegalovirus
cCMV	congenital Cytomegalovirus infection
DBS	dried blood spots
Mya	million years ago
IE	immediate early genes
Abs	antibodies
Ags	antigens
dNK	decidual NK cells
KIRs	killer cell immunoglobulin-like receptors
APC	antigen presenting cell
DC	dendritic cell
LTI	long-term impairments
AF	amniotic fluid
MRI	magnetic resonance imaging
CSF	cerebrospinal fluid
CNS	central nervous system
CT	computed tomography
cCMV+	children with congenital CMV
cCMV-	children without congenital CMV
TRECs	TCR excision circles
KRECs	Igκ-deleting recombination excision circles
cj intronRSS-Kde	intronRSS-Kde coding joints
C16	palmytoilcarnitine
17OHP	17-α-hydroxyprogesteron
T4	thyroxine
AF	amniotic fluid
BIOT	biotinidase activity
MS/MS	Tandem Mass Spectrometry
dNK	decidual natural killer cells
mm	mismatches
NIMAs	non-inherited maternal antigens
IPA	inherited paternal antigen
Ags	Antigens
HLA-G deletion	HLA-G del/del
sHLA-G	soluble HLA-G
RPM	reads per million

ENGLISH SUMMARY

Cytomegalovirus (CMV) infection is a worldwide common infection that in a considerable proportion of individuals remains unnoticed. However, when the virus infects the fetus via the placenta during pregnancy, it can cause severe disease. The congenital CMV infection (cCMV) can induce a variety of clinical manifestations at birth (symptoms at birth), and of permanent long-term impairments (LTI). These clinical signs can also vary in severity, e.g. in symptomatic neonates they can range from elevated liver enzymes to microcephaly (having a smaller than normal head). The permanent long-term disabilities (ranging from neurological to visual impairments) can be progressive or reveal themselves later in childhood, for example late onset hearing loss, and in this context a retrospective diagnosis may be challenging. Importantly, of the total of infected neonates at birth, 13% are symptomatic at birth, and half of them will develop LTI. However, 13% of the asymptomatic neonates will still develop the same LTI; and since this group is much bigger than the symptomatic group, the majority of clinical cases come from the asymptomatic neonates. The latter is where the cCMV gets easily unnoticed because you do not see anything suspicious at birth. Summarizing, not all infected foetuses will have symptoms at birth or will develop long-term disabilities. At this moment, there is no prognostic marker available to anticipate which neonates, and when, will develop LTI, nor which type of disability. cCMV can occur at any time during pregnancy, and usually when it occurs early in pregnancy it can cause more severe disease because the fetus is still developing. cCMV can occur in mothers after their first infection with CMV during pregnancy (who were previously seronegative) or who were previously infected with CMV (seropositive). In the latter, the vertical transmission rate (i.e. CMV transmission from the mother to the fetus) is usually lower than in the seronegative pregnant women, because of the pre-existing immunity to CMV, but it is not clear whether this immunity also protects against severe clinical problems. These two variables, trimesters of maternal infection and pre-existing immunity, complicate studies on cCMV pathogenesis in the general population.

The aim of this thesis was to get new insights into cCMV pathogenesis, and to identify prognostic markers for short- and long-term clinical outcome, and correlates of protection for vaccine development. For this purpose, a retrospective nation-wide cohort of congenitally infected children and controls (i.e. non-infected children) was used. All children were born in The Netherlands in 2008, and dried blood spots (DBS) were used to diagnose cCMV. DBS are obtained by spotting whole blood from the heels of the neonate within one week after birth. These cards are routinely used for the screening of the rare genetic metabolic disorders for which a clinical, life-saving, intervention is available. DNA was extracted from these cards, and the presence of CMV DNA was evaluated in order to diagnose cCMV. Additionally, by quantification of CMV DNA, the viral load (which gives an indication on how much virus is present in the body) was determined. Of the children in this cohort, clinical data from birth till 6 years of age were available. Another specimen collected from this cohort, from both mothers and children, was represented by buccal swabs. DNA was extracted from buccal swabs for several genetic studies. In order to reach our goals, the following biomarkers

were evaluated and related to CMV viral load, symptoms at birth and LTI development at 6 years of age:

- markers for B cells, αβ and γδ T cells in DBS;
- gene expression profiles with specific attention to the immune system and inflammation pathways;
- data on essential amino acids, hormones, carnitines and enzymes in DBS;
- HLA (human leukocyte antigens) typing on buccal swabs of mothers and children with cCMV.

In the following paragraphs, the main findings of all chapters are presented. First, the above mentioned biomarkers in relation to cCMV infection itself and the viral load in DBS are presented. Then, the biomarkers in relation to symptoms at birth and LTI development.

Congenital CMV infection

In order to study the effect of cCMV on different markers, we compared the biomarkers in DBS or buccal swabs in children with cCMV to those without cCMV (our control group). In our cohort, cCMV resulted in a reduced production of αβ T cells in the thymus, an increased number of γδ T cells, and increased production of B cells (**Chapter 2**). Additionally, cCMV induced exhaustion of T cells (i.e. a general state of cellular dysfunction usually induced by chronic infections and exposure to high viral loads) (**Chapter 4**). Of these markers, only the production of αβ T cells was not associated with CMV viral load. In **Chapter 4**, the gene expression profile on the DBS showed that gene expression of genes related to the innate immune response and NK cell activation was higher in children with a higher CMV viral load. The innate immune system, NK cells and γδ T cells may play an important role in controlling CMV in the foetus, where the CMV-specific responses are still developing. Furthermore, a specific HLA of Class II in children (i.e. molecule that presents CMV components to immune cells), i.e. HLA-DRB1*04, was associated with a better CMV viral control (**Chapter 6**). This positive effect on viral control may rely on the fact that a HLA Class II supports both arms of the immune response, cytotoxic and antibody responses. Furthermore, cCMV did not affect the neonatal metabolism in DBS, but a reduced level of essential aminoacids (components of proteins) was found in the subgroup of congenitally infected neonates that was premature (i.e. birth before the 37 weeks of gestation) compared to the prematurely born children without cCMV (**Chapter 3**). Although cCMV did not affect the neonatal metabolism, a higher CMV viral load induced an increase of C16 (**Chapter 3**). C16 is a fatty acid and the precursor to longer chain fatty acids, for which the outer structure of CMV is enriched. Therefore, the increase of C16 in the high viral load group may simply reflect the increased viral burden.

Symptoms at birth

The different markers were also studied in relation to the presence of symptoms at birth, by comparing the markers in the cCMV group with symptoms to the cCMV group without. The only

markers related to symptoms at birth were found while studying the HLA-types of both mothers and children, with their specific combination. In **Chapter 5** and **Chapter 6** we assessed the role of HLA Class I and Class II, as well as the receptors for NK cells of the mothers, in relation to cCMV clinical outcome. We found that mothers with a specific HLA-G deletion/deletion genotype or mothers homozygous for HLA-C1, as well as mother-child pairs with HLA-E and HLA-C mismatches were associated with symptomatic disease (**Chapter 5**). The mothers that have the HLA-G deletion/deletion genotype probably have higher levels of the HLA-G protein that has immunosuppressive properties. The immunosuppression may lead to less viral control upon CMV infection during pregnancy and may lead to a higher viral burden of the placenta. A HLA mismatch exists when the mother does not have the same genetic HLA information as the fetus has, because some of the genetic HLA information derives from the father, which may be different from that of the mother. In **Chapter 5,** we demonstrated that in case of HLA-E or HLA-C mismatch the chance of a child with symptoms at birth was higher. A mismatch of HLA, such as HLA-E and HLA-C that are expressed in the placenta, may lead to maternal T cells that are capable of attacking the fetal tissue. Normally, this process is suppressed, but in case of a placental CMV infection, this balance may be disturbed leading to a T cell reaction towards the placenta and the fetus. Furthermore, HLA matching is necessary for an adequate immune response to virus-infected cells. Therefore, a HLA mismatch may lead to a worse immune reaction and less control of the viral infection, with higher placental and possibly fetal viral loads as a result. Finally, one of the most abundant cells in the placenta is the NK cell, which belong to the innate immunity and therefore react rapidly upon activation. These cells have the peculiar characteristic of having several activating and inhibitory receptors whose combination defines the degree of NK cells activation. The HLA-C molecules can bind to these receptors with different degrees of strength. In our cohort, in the group with symptoms at birth, the mothers more frequently missed the inhibitory type of HLA-C, and therefore the activating signals towards NK cells may prevail contributing to the already increased placental inflammation (**Chapter 5).** In **Chapter 6** the HLA-types that are not expressed in the placenta were studied. For these HLA-types no association with symptoms at birth was found. Summarizing, symptoms at birth appear to be related to genetic markers that contribute to high CMV viral load at maternal and placental level, due to a lack of viral control, and to a higher level of placental inflammation.

Long-term impairments

In three chapters biomarkers are described that seem to be associated with LTI at 6 years of age. In **Chapter 2**, infected children that developed LTI had lower numbers of B cells. In addition, we found higher percentage of HLA-C non-inherited maternal antigens (NIMA) in those infected children that developed LTI (**Chapter 5**). The fetus is exposed to antigens that he/she did not inherit, the so-called NIMA, because during pregnancy and breast-feeding there is a mutual exchange of cells between mother and child. Small numbers of maternal cells seed in several fetal organs and persist at least until adulthood. However, instead of the initiation of a fetal immune response against these "foreign" maternal antigens, the fetus develops a long-lasting tolerance (NIMA effect). The NIMA effect is beneficial in the context of transplantation because it can improve graft survival.

However, during infections the NIMA effect may induce an indirect tolerance to the infection as well. Consequently an indirect tolerance to the CMV infection may induce an uncontrolled viral replication throughout childhood. Furthermore, in **Chapter 4,** we show that in children that did not develop LTI there was a higher expression of anti-inflammatory markers. This further suggests that part of the pathogenesis of LTI development can be attributable to an uncontrolled infection and inflammation.

The aim of this thesis was to identify prognostic markers for short- and long-term clinical outcome and correlates of protection for vaccine development. We did find some interesting biomarkers that, if confirmed in other cohorts, could be potential candidates for such goals. However, sometimes the differences were too small, and a striking biomarker was not found. On the other hand, we did find some clues towards a better understanding of cCMV pathogenesis (i.e. biological mechanisms that leads to disease). Understanding cCMV pathogenesis is fundamental for several reasons. First of all, it would help future research on prognostic markers. Second of all, it would support the introduction of innovative clinical strategies to prevent, or at least positively affect, the short- and long-term clinical outcome because we would understand the mechanisms for the progressive and permanent damage. Indeed, the way to a licensed vaccine is way too long to just patiently wait for it. However, in the end, vaccination should be the main goal in cCMV research because it could be the ultimate solution for prevention of vertical transmission during pregnancy, or disease development in the child. Finally, the identification of the aforementioned biomarker could help the future research for vaccine development.

To conclude, from the research presented in this thesis, several issues have emerged that should be taken into account in future research. The majority of congenitally infected neonates are asymptomatic at birth and have a good prognosis for a normal development, therefore the comparison of their immunological state with that of the symptomatic infected neonates with a worse long-term outcome, as well as those asymptomatic that develop the same LTI, is essential to determine what is happening in the children who are affected by cCMV. While doing that, we should also define new clinical strategies, and also study whether the current clinical strategies are beneficial for those asymptomatic neonates that develop the same LTI, and whether for this group the benefits of for example antiviral treatment outweigh the side effects. However, an even earlier goal should be the standardization of the definitions of symptomatic disease and LTI development without which no reliable conclusion can be made on cCMV pathogenesis.

SAMENVATTING

Cytomegalovirus (CMV) infectie is een wereldwijd voorkomende infectie die bij het merendeel van de personen onopgemerkt blijft. Wanneer het virus echter tijdens de zwangerschap het ongeboren kind (foetus) via de placenta infecteert, kan het ernstige ziekte veroorzaken. Deze congenitale, of aangeboren, CMV infectie (cCMV) kan een verscheidenheid aan klinische manifestaties veroorzaken bij de geboorte (symptomen bij de geboorte) en op de lange termijn, de zogenaamde lange termijn gevolgen (LTG). De klinische symptomen kunnen ook variëren in ernst, b.v. bij symptomatische pasgeborenen kunnen ze variëren van verhoogde leverenzymen tot microcefalie (een te klein hoofd). De permanente lange termijn beperkingen (variërend van problemen met het zicht tot ernstige neurologische problemen) kunnen deels progressief zijn of zich later in de kindertijd openbaren, bijvoorbeeld het later ontstaan van gehoorverlies. In dat geval kan een retrospectieve diagnose een uitdaging zijn. Belangrijk is dat van het totaal aantal geïnfecteerde pasgeborenen 13% symptomatisch is bij de geboorte en de helft van deze kinderen LTG zal ontwikkelen. Echter, ook 13% van de asymptomatische pasgeborenen zal dezelfde LTG ontwikkelen; en aangezien deze groep veel groter is dan de symptomatische groep, zijn de meeste kinderen met lange termijn problemen afkomstig uit de groep die asymptomatisch bij geboorte was. In dit laatste geval wordt cCMV dus makkelijk gemist omdat er bij de geboorte geen verdenking ontstaat. Samenvattend, zullen niet alle geïnfecteerde kinderen bij de geboorte symptomen vertonen of langdurige beperkingen ontwikkelen. Op dit moment is er geen prognostische marker beschikbaar om te voorspellen welke neonaten LTG zullen hebben, en wanneer, en welk type beperking ze zullen krijgen. cCMV kan op elk moment tijdens de zwangerschap optreden, maar de gevolgen zijn ernstiger wanneer de infectie vroeg in de zwangerschap optreedt, omdat de foetus zich dan nog ontwikkelt. cCMV kan vóórkomen bij moeders na hun eerste infectie met CMV tijdens de zwangerschap (moeders die eerder seronegatief waren) of bij moeders die al eerder geïnfecteerd waren met CMV (dus seropositief waren). Bij de laatste groep is de verticale transmissie kans (dat wil zeggen de kans dat er CMV-overdracht plaatsvindt van de moeder naar de foetus) meestal lager dan bij de seronegatieve zwangere vrouwen, vanwege de reeds bestaande immuniteit tegen CMV, maar het is niet duidelijk of deze immuniteit ook beschermt tegen ernstige klinische problemen. Deze twee variabelen, trimester van maternale infectie en reeds bestaande immuniteit, bemoeilijken studies over cCMV-pathogenese in de algemene populatie.

Het doel van dit proefschrift was om nieuwe inzichten te verkrijgen in cCMV-pathogenese en om prognostische markers te identificeren voor korte- en lange termijn klinische uitkomsten. Hieraan gerelateerd zou de studie een maat voor bescherming tegen CMV kunnen opleveren, welke te gebruiken bij vaccinontwikkeling. Voor dit doel werd een retrospectief Nederlands cohort van kinderen met cCMV en CMV-negatieve controles (niet-geïnfecteerde kinderen) gebruikt. Alle kinderen zijn in 2008 in Nederland geboren en bloed op het hielprikkaartje werd gebruikt om cCMV te diagnosticeren. De hielprikkaartjes worden verkregen door binnen een week na de geboorte bloed uit de hiel van de pasgeborene op een filterpapier te druppelen. Deze hielprikkaarten worden routinematig gebruikt voor het screenen op zeldzame genetische en metabole aandoeningen waarvoor een levensreddende interventie beschikbaar is. Deze kaarten

zijn getest op de aanwezigheid van CMV-DNA om cCMV te diagnosticeren. Bovendien werd, door het meten van de hoeveelheid CMV-DNA, de "virale load" bepaald (een maat voor hoeveel virus in het lichaam aanwezig is). Van de kinderen in dit cohort waren klinische gegevens beschikbaar vanaf de geboorte tot de leeftijd van 6 jaar. Verder is van zowel moeders als kinderen een beetje wangslijmvlies afgenomen. DNA uit wangslijmvlies is gebruikt voor verschillende genetische studies. Voor de studie werden de volgende biomarkers onderzocht in relatie tot cCMV, de CMV-virale load, symptomen bij de geboorte en LTG tot de leeftijd van 6 jaar:

- Markers voor B-cellen, αβ- en γδ-T-cellen in de hielprikkaart
- Genexpressie profielen in het bijzonder in relatie tot het immuunsysteem en ontstekingsreacties in de hielprikkaart
- Gegevens over essentiële aminozuren, hormonen, carnitines en enzymen in de hielprikkaart
- HLA (humane leukocyten antigenen) op wangslijmvlies van moeders en kinderen

In de volgende paragrafen worden de belangrijkste bevindingen van alle hoofdstukken weergegeven. Eerst worden de bovengenoemde biomarkers met betrekking tot cCMV-infectie zelf en de virale load in de hielprikkaart beschreven. Vervolgens, de biomarkers in relatie tot symptomen bij de geboorte en LTG-ontwikkeling.

Congenitale CMV infectie

Om het effect van cCMV op de verschillende markers te bestuderen, hebben we de biomarkers in de hielprikkaart of wangslijmvlies bij kinderen met cCMV vergeleken met die bij kinderen zonder cCMV (onze controlegroep). In ons cohort was in de cCMV groep een verminderde productie van αβ T-cellen in de thymus aantoonbaar, een verhoogd aantal γδ T-cellen en verhoogde productie van B-cellen (**hoofdstuk 2**). Bovendien was bij cCMV uitputting van T-cellen te zien (d.w.z. een algemene toestand van cellulaire dysfunctie die gewoonlijk wordt gezien bij chronische infecties en blootstelling aan hoge virale loads) (**hoofdstuk 4**). Van deze markers was alleen de productie van αβ T-cellen niet geassocieerd met CMV virale load. In **hoofdstuk 4** toonde het genexpressieprofiel op de hielprikkaart een hogere expressie van genen gerelateerd aan de aangeboren immuunrespons en NK-cel activatie bij kinderen met een hogere CMV virale load. Het aangeboren immuunsysteem, waaronder NK-cellen en γδ T-cellen, kan een belangrijke rol spelen bij het onder controle houden van CMV in het ongeboren kind, waar de CMV-specifieke immuunreactie zich nog moet ontwikkelen. Verder was een specifiek HLA van klasse II bij kinderen (d.w.z. een molecuul dat CMV-componenten presenteert aan immuun cellen), namelijk HLA-DRB1*04, geassocieerd met een betere CMV virus controle (**hoofdstuk 6**). Dit positieve effect op virus controle kan berusten op het feit dat een HLA klasse II beide armen van de specifieke immuunrespons, de cytotoxische en antilichaamreacties, ondersteunt. cCMV had geen invloed op het neonatale metabolisme in de hielprikkaart, maar een verlaagde hoeveelheid essentiële aminozuren (bestanddelen van eiwitten) werd gevonden in de subgroep van te vroeg geboren pasgeborenen met cCMV (geboren vóór de 37 weken zwangerschap) in vergelijking met te vroeg geboren kinderen zonder cCMV (**hoofdstuk 3**). Hoewel cCMV het neonatale metabolisme niet beïnvloedde, induceerde een

hogere CMV virale load een toename van C16 (**hoofdstuk 3**). C16 is een vetzuur en de voorloper van vetzuren met een langere keten, die ook voorkomen in de envelop van CMV. Daarom kan de toename van C16 in de groep met hogere virale load het gevolg zijn van hogere virale replicatie.

Symptomen bij geboorte

De verschillende markers werden ook bestudeerd in relatie tot de aanwezigheid van symptomen bij de geboorte, door de markers in de cCMV-groep met symptomen te vergelijken met die in de groep zonder symptomen. De enige markers gerelateerd aan symptomen bij de geboorte werden gevonden tijdens het bestuderen van de HLA-types van zowel moeders als kinderen, en specifieke moeder-kind combinaties. In **hoofdstuk 5** en **hoofdstuk 6** hebben we de rol van HLA Klasse I en Klasse II, als ook van NK-cel receptoren bij de moeder onderzocht in relatie tot de klinische uitkomst van cCMV. We vonden dat een specifiek HLA-G-deletie/deletie genotype bij moeder, homozygotie voor HLA-C1 bij moeder, en moeder-kind HLA-E en HLA-C mismatches geassocieerd waren met symptomatische ziekte (**hoofdstuk 5**). De moeders met het HLA-G-deletie/deletie genotype hebben waarschijnlijk hogere niveaus van het HLA-G-eiwit, dat immunosuppressieve eigenschappen heeft. De immunosuppressie kan leiden tot minder controle van het virus na CMV-infectie tijdens de zwangerschap en kan leiden tot een hogere virale load in de placenta. Er is sprake van een HLA-mismatch wanneer de moeder niet dezelfde genetische HLA-informatie heeft als het ongeboren kind, omdat een deel van de genetische HLA-informatie afkomstig is van de vader, die anders kan zijn dan die van de moeder. In **hoofdstuk 5** hebben we aangetoond dat in het geval van HLA-E of HLA-C mismatch de kans op een kind met symptomen bij de geboorte hoger was. Een mismatch van HLA, zoals van HLA-E en HLA-C die aanwezig zijn in de placenta, kan leiden tot maternale T-cellen die gericht zijn tegen foetaal weefsel. Normaal gesproken wordt dit proces onderdrukt, maar in het geval van een CMV-infectie van de placenta kan dit evenwicht verstoord worden, hetgeen zou kunnen leiden tot een T-celreactie tegen de placenta en de foetus. Verder is HLA-matching noodzakelijk voor een adequate immuunrespons op virus geïnfecteerde cellen. Daarom kan een HLA-mismatch leiden tot een slechtere immuunreactie en minder controle van de virale infectie, met als gevolg een hogere virale load in placenta en mogelijk ook de foetus. Ten slotte is één van de meest voorkomende cellen in de placenta de NK-cel, die tot de aangeboren immuniteit behoort en daarom snel reageert bij activatie. Deze cellen hebben het bijzondere kenmerk dat zij zowel verschillende activerende en remmende receptoren bezitten waarbij de combinatie bepaalt in welke mate de NK-cellen geactiveerd worden. De HLA-C-moleculen kunnen in verschillende mate aan deze receptoren binden. In de groep met symptomen bij de geboorte, misten de moeders vaker het remmende HLA-C type. Daarom zouden de activerende signalen bij NK-cellen de overhand kunnen krijgen, hetgeen bijdraagt aan een (nog verder) toegenomen inflammatie van de placenta (**hoofdstuk 5**). In **hoofdstuk 6** werden de HLA-types bestudeerd die niet in de placenta tot expressie komen. Voor deze HLA-types werd geen verband gevonden met symptomen bij de geboorte. Samenvattend lijken de symptomen bij de geboorte verband te houden met genetische markers die bijdragen aan een hoge CMV virale load bij de moeder en in de placenta vanwege een gebrek aan virale controle en met een sterkere mate van placentaire ontsteking.

Lange-termijn gevolgen

In drie hoofdstukken worden biomarkers beschreven die geassocieerd lijken te zijn met LTG op 6-jarige leeftijd. In **hoofdstuk 2** hadden geïnfecteerde kinderen die LTG hadden een lager aantal B-cellen. Bovendien vonden we een hoger percentage van HLA-C niet-overerfde maternale antigenen (NIMA) in die geïnfecteerde kinderen mét LTG (**hoofdstuk 5**). Het kind wordt blootgesteld aan antigenen die hij niet heeft geërfd, de zogenaamde NIMA, omdat er tijdens de zwangerschap en borstvoeding een wederzijdse uitwisseling van cellen tussen moeder en kind plaatsvindt. Kleine aantallen maternale cellen nestelen zich in verschillende foetale organen en blijven daar tenminste tot de volwassen leeftijd is bereikt. In plaats van een immunologische reactie tegen deze "vreemde" maternale antigenen, ontwikkelt de foetus een langdurige tolerantie (NIMA-effect). Het NIMA-effect is gunstig in de context van transplantatie omdat het de overleving van transplantaten kan verbeteren. Tijdens infecties kan het NIMA-effect echter ook een indirecte tolerantie voor de verwekker van de infectie veroorzaken. Indirecte tolerantie voor de CMV-infectie zou een ongecontroleerde virale replicatie kunnen veroorzaken gedurende de kindertijd. In **hoofdstuk 4** laten we tenslotte zien dat bij kinderen die geen LTG hebben een hogere expressie van anti-inflammatoire markers was. Dit suggereert dat een deel van de pathogenese van lange termijn schade kan worden toegeschreven aan een ongecontroleerde infectie en ontsteking.

Het doel van dit proefschrift was om prognostische markers te identificeren voor korte en lange termijn klinische uitkomsten en correlaten van bescherming voor de ontwikkeling van vaccins. We hebben enkele interessante biomarkers gevonden die, indien bevestigd in andere cohorten, potentiële kandidaten voor dergelijke doelen zouden kunnen zijn. Soms waren de verschillen tussen de groepen te klein en er was niet één biomarker die eruit sprong. Aan de andere kant hebben de bevindingen wel geleid tot een beter begrip van de cCMV-pathogenese (d.w.z. de biologische mechanismen die tot ziekte leiden). Het begrijpen van cCMV-pathogenese is om verschillende redenen relevant. Allereerst kan dit toekomstig onderzoek naar prognostische markers helpen. Ten tweede, als we de mechanismen voor de progressieve en permanente schade beter begrijpen, kan dit de toepassing van innovatieve klinische strategieën ondersteunen waarmee de korte- en lange termijn klinische uitkomst positief beïnvloed kan worden. Uiteindelijk zou een geregistreerd CMV vaccin het ultieme doel van cCMV-onderzoek zijn. Vaccinatie zou verticale transmissie tijdens de zwangerschap kunnen voorkomen en cCMV geassocieerde ziekte bij het kind. De identificatie van de bovengenoemde biomarker zou ook kunnen helpen bij toekomstig onderzoek naar de ontwikkeling van vaccins.

Tot slot, uit het onderzoek zoals beschreven in dit proefschrift, zijn verschillende uitdagingen naar voren gekomen waarmee bij toekomstig onderzoek rekening gehouden kan worden. De meerderheid van de pasgeborenen met cCMV is asymptomatisch bij de geboorte en heeft een goede prognose en een normale ontwikkeling. Daarom is de vergelijking van de immunologische kenmerken van deze kinderen met die van symptomatische pasgeborenen mét lange termijn schade, als ook met die van asymptomatisch pasgeborenen met dezelfde LTG essentieel om te bepalen welke factoren bepalend zijn voor lange termijn schade. Ondertussen moeten we nieuwe klinische strategieën ontwerpen, en onderzoeken of de huidige klinische strategieën van

nut zijn voor die asymptomatische pasgeborenen. Bij deze groep moet bijvoorbeeld onderzocht worden of de voordelen van antivirale behandeling opwegen tegen de bijwerkingen. Daarnaast is het van belang om te komen tot standaardisatie van de definities van symptomatische ziekte en LTG-ontwikkeling, zonder welke geen betrouwbare conclusie kan worden getrokken over cCMV-pathogenese.

ITALIAN SUMMARY

L'infezione da Citomegalovirus (CMV) e' un'infezione diffusa in tutto il mondo che, in una notevole proporzione di individui, rimane inosservata. Tuttavia, il virus puo' causare gravi manifestazioni quando, durante la gravidanza, infetta il feto passando attraverso la placenta. L'infezione congenita da CMV (cCMV) puo' indurre una varieta' di manifestazioni cliniche alla nascita (sintomi alla nascita), e di danni permanenti a lungo termine (LTI). Tali segni clinici possono anche variare in gravita', per esempio in neonati sintomatici alla nascita possono variare da enzimi epatici elevati a microcefalia (testa piu' piccola del normale). Le disabilita' permanenti a lungo termine (che vanno da danni neurologici a danni visivi) possono essere progressivi o manifestarsi tardi nell'infanzia, come per esempio la perdita dell'udito a esordio ritardato, ed in questo contesto una diagnosi retrospettiva potrebbe essere difficile. In particolare, del totale di neonati infetti alla nascita, il 13% e' sintomatico alla nascita, e meta' di loro sviluppa LTI. Tuttavia, il 13% dei neonati asintomatici alla nascita sviluppera' gli stessi LTI; e dal momento che questo gruppo e' molto piu' grande del gruppo di neonati sintomatici alla nascita, la maggior parte dei casi clinici di LTI deriva dal gruppo degli asintomatici. Ed e' proprio in quest'ultimo gruppo che la cCMV viene facilmente inosservata perche' non si vede nulla alla nascita. Riassumendo, non tutti i feti infetti avranno sintomi alla nascita o svilupperanno disabilita' a lungo termine. In questo momento, non c'e' nessun marcatore prognostico disponibile che possa predire quale neonato sviluppera' LTI, e quando o quale tipo di disabilita' avra'. La cCMV puo' avvenire in qualsiasi momento durante la gravidanza, e di solito quando avviene durante le fasi iniziali della gravidanza puo' causare dei danni piu' severi perche' il feto si sta ancora sviluppando. La cCMV puo' avvenire quando le madri si infettano per la prima volta durante la gravidanza (che erano sieronegative) oppure che erano gia' infette con CMV (sieropositive). Nell'ultimo caso, data la pre-esistente immunita' al CMV, la velocita' di trasmissione verticale (cioe' la trasmissione del CMV dalla madre al feto) e' di solito piu' bassa che in donne incinte sieronegative, ma non e' chiaro se tale immunita' protegga anche da diversi problemi clinici severi. Queste due variabili, il trimestre di infezione materna e la pre-esistente immunita', complicano gli studi sulla patogenesi di cCMV nella popolazione generale.

Lo scopo di questa tesi era di acquisire nuove conoscenze sulla patogenesi di cCMV, di identificare marker prognostici per l'esito clinico a breve e lungo termine, e correlati di protezione per lo sviluppo del vaccino. A tal proposito, e' stata usata. una coorte retrospettiva su scala nazionale di bambini congenitamente infetti e di controlli (cioe' bambini non infetti). Tutti questi bambini sono nati nei Paesi Bassi nel 2008, e per diagnosticare cCMV sono stati utilizzati campioni di sangue essiccato (DBS). I DBS sono stati ottenuti da sangue intero dal tallone dei neonati entro una settimana dalla nascita. Questi campioni sono di solito usati di routine per lo screening delle malattie genetiche rare per le quali sono disponibili interventi clinici salva vita. Il DNA e' stato estratto da questi campioni, e la presenza del DNA virale e' stata utilizzata per diagnosticare cCMV. Inoltre, mediate quantificazione del DNA virale e' stata determinata la carica virale (che da' un'indicazione della quantita' di virus presente nel corpo). Dei bambini di questa coorte, erano disponibili i dati clinici dalla nascita fino a 6 anni d'eta'. Inoltre, sono stati ottenuti ulteriori campioni, sia dalle madri che dai bambini, sottoforma di tamponi buccali. Il DNA e' stato estratto dai tamponi buccali per

eseguire diversi studi genetici. Al fine di raggiungere i nostri obiettivi, si sono studiati i seguenti biomarkers in relazione alla carica virale, ai sintomi alla nascita e allo sviluppo di LTI a 6 anni d'eta':

- markers per cellule B e cellule T, sia αβ che γδ, nei DBS;
- i profili di espressione genica con particolare attenzione ai pathways del sistema immunitario e infiammatorio;
- dati sugli aminoacidi essenziali, ormoni, carnitine ed enzimi su DBS;
- tipizzazione HLA (antigene leucocitario umano) sui tamponi buccali sia delle madri che dei bambini con cCMV.

Nei seguenti paragrafi sono presentati i principali risultati di tutti i capitoli. Prima di tutto, sono presentati i sopracitati biomarkers in relazione all'infezione cCMV e alla carica virale. Poi, tali biomarkers sono stati presentati in relazione ai sintomi alla nascita ed allo sviluppo di LTI.

Infezione congenita da CMV

Al fine di studiare l'effetto di cCMV sui diversi markers, abbiamo comparato i biomarkers dai DBS o dai tamponi buccali in bambini con cCMV a quelli senza cCMV (il nostro gruppo di controllo). Nella nostra coorte, cCMV e' risultato in una riduzione della produzione delle cellule T αβ nel timo, in un aumentato numero delle cellule T γδ, e in un'aumentata produzione delle cellule B (**Capitolo 2**). Inoltre, cCMV ha provocato l'esaustione delle cellule T (cioe' uno stato generale di disfunzione cellulare solitamente indotto dalle infezioni croniche e dall'esposizione ad alte cariche virali) (**Capitolo 4**). Di questi markers, solo la produzione delle cellule T αβ non era associata alla carica virale. Nel **Capitolo 4**, il profilo di espressione genica su DBS ha mostrato che l'espressione genica relativa alla risposta immunitaria innata e all'attivazione delle cellule NK era piu' elevata nei bambini con una carica virale elevata. Il sistema immunitario innato, le cellule NK e le cellule T γδ potrebbero svolgere un ruolo nel controllare CMV nel feto, dove le risposte CMV-specifiche si stanno ancora sviluppando. Inoltre, lo specifico HLA di classe II HLA-DRB1*04 (cioe' una molecola che presenta componenti virali alle cellule immunitarie) e' risultata correlata ad un miglior controllo virale nei bambini (**Capitolo 6**). Tale effetto positivo sul controllo virale potrebbe dipendere dal fatto che le molecole HLA di classe II supportano entrambe le braccia della risposta immunitaria, citotossica e anticorpale. Inoltre, cCMV non ha influenzato il metabolismo neonatale su DBS, ma un ridotto livello di aminoacidi essenziali (componenti delle proteine) e' stato trovato nel sottogruppo di bambini infetti nati prematuri (cioe' prima delle 37 settimane di gestazione) comparata al sottogruppo di bambini senza cCMV nati prematuri (**Capitolo 3**). Sebbene cCMV non abbia influenzato il metabolismo neonatale, una carica virale elevata ha indotto un aumento di C16 (**Capitolo 3**). C16 e' un acido grasso e precursore di acidi grassi a catena piu' lunga, per il quale la struttura esterna virale e' arricchita. Pertanto, l'aumento di C16 nel gruppo con carica virale elevata potrebbe semplicemente riflettere l'aumentato onere virale a livello cellulare.

Sintomi alla nascita

I diversi markers sono stati studiati anche in relazione alla presenza di sintomi alla nascita, comparandoli tra il gruppo di bambini infetti con sintomi alla nascita e bambini infetti privi di sintomi. Gli unici markers associati a sintomi alla nascita sono stati trovati studiando le molecole HLA di entrambe madri e bambini, con la loro specifica combinazione. Nel **Capitolo 5** e **Capitolo 6** abbiamo valutato il ruolo delle molecole HLA di Classe I e Classe II, cosi' come dei recettori delle cellule NK materne, in relazione all'esito clinico. E' emerso che madri con lo specifico genotipo HLA-G delezione/delezione, o madri omozigoti per HLA-C1, cosi' come coppie madre-figlio con discordanze per le molecule HLA-E ed HLA-C, fossero associate con sintomi alla nascita (**Capitolo 5**). Le madri con un genotipo HLA-G delezione/delezione probabilmente hanno livelli piu' elevati della proteina HLA-G che ha proprieta' immunosoppressive. L'immunosoppressione potrebbe indurre un ridotto controllo virale sull'infezione durante la gravidanza, e potrebbe indurre un aumentato carico virale a livello della placenta. Una discordanza HLA si ha quando la madre non ha la stessa informazione genetica del feto, perche' alcune delle informazioni genetiche derivano dal padre e potrebbero essere diverse da quella della madre. Nel **Capitolo 5**, abbiamo dimostrato che in caso di discordanza HLA-E e HLA-C le chance di un bambino con sintomi alla nascita erano piu' alte. Una discordanza di HLA, come HLA-E e HLA-C che sono molecole espresse dalla placenta, potrebbe indurre la formazione di cellule T materne capaci di attaccare i tessuti fetali. Normalmente, tale processo e' soppresso, ma in caso di infezione della placenta da CMV, tale equilibrio potrebbe essere alterato ed indurre reazioni delle cellule T contro la placenta ed il feto. Inoltre, la concordanza HLA e' necessaria per un'adeguata risposta immunitaria contro le cellule infette. Pertanto, una discordanza HLA potrebbe indurre una peggior reazione immunitaria e un minor controllo dell'infezione, con una conseguente carica virale placentale e fetale piu' elevata. Infine, una delle popolazioni cellulari piu' abbondanti a livello placentare e' rappresentata dalle cellule NK, che appartengono all'immunita' innata, e pertanto possono reagire rapidamente dopo attivazione. Queste cellule hanno la peculiare caratteristica di avere diversi recettori attivatori e inibitori la cui combinazione definisce il grado di attivazione delle cellule NK. Le molecule HLA-C possono legare tali recettori con diversi gradi di forza. Nella nostra coorte, le madri con neonati sintomatici alla nascita piu' frequentemente non avevano il tipo inibitorio di HLA-C, e pertanto i segnali attivatori sulle cellule NK potrebbero prevalere contribuendo all'infiammazione placentare gia' elevata (**Capitolo 5**). Nel **Capitolo 6** sono state studiate le molecole HLA non espresse dalla placenta. Per queste molecole non sono state trovate associazioni con sintomi alla nascita. Riassumendo, i sintomi alla nascita sembrerebbero essere collegati a fattori genetici che contribuirebbero ad aumentare la carica virale materna e palcentare, dovuti ad un ridotto controllo dell'infezione, e ad un'aumentata infiammazione della placenta.

Disabilita' a lungo termine

In tre capitoli sono stati descritti biomarkers che sembrerebbero essere associati con LTI a 6 anni d'eta'. Nel **Capitolo 2**, i bambini infetti che hanno sviluppato LTI hanno mostrato un numero ridotto di cellule B. Inoltre, abbiamo trovato un'aumentata percentuale di antigeni HLA-C materni non ereditati

(NIMA) in quei bambini infetti che hanno sviluppato LTI (**Capitolo 5**). Il feto e' esposto ad antigeni che lui/lei non ha ereditato, il cosiddetto NIMA, perche' durante la gravidanza e l'allattamento c'e' uno scambio mutuale di cellule tra la madre ed il bambino. Diverse cellule materne si collocano in diversi organi fetali e persistono almeno almeno fino all'eta' adulta. Tuttavia, invece che sviluppare una risposta immunitaria fetale contro questi antigeni materni "estranei", il feto sviluppa una tolleranza di lunga durata (effetto NIMA). L'effetto NIMA e' vantaggioso nel contesto del trapianto perche' puo' migliorare la sopravvivenza dell'organo. Tuttavia, durante le infezioni l'effetto NIMA potrebbe indurre una tolleranza indiretta anche all'infezione. Di conseguenza, durante tutta l'infanzia, una tolleranza indiretta all'infezione potrebbe indurre un'incontrollata replicazione virale. Inoltre, nel **Capitolo 4**, nei bambini che non sviluppano LTI c'era una piu' elevata espressione di markers anti infiammatori. Questo suggerisce ulteriormente che parte della patogenesi dello sviluppo di LTI puo' essere attribuibile ad un'incontrollata infezione ed infiammazione.

Lo scopo di questa tesi era di identificare marker prognostici per l'esito clinico a breve e lungo termine, e correlati di protezione per lo sviluppo del vaccino. Abbiamo trovato alcuni biomarkers interessanti che, se confermati in altre coorti, potrebbero essere potenziali candidati per tale scopo. Tuttavia, talvolta le differenze erano troppo piccole che non e' stato trovato un marker determinante. D'altro canto, abbiamo trovato alcuni indizi per una migliore comprensione della patogenesi di cCMV (cioe' i meccanismi biologici che conducono alla malattia). Comprendere la patogenesi di cCMV e' fondamentale per diverse ragioni, prima di tutto aiuterebbe la futura ricerca di marker prognostici, in secondo luogo supporterebbe l'introduzione di strategie cliniche innovative per prevenire, o perlomeno, influenzare positivamente l'esito clinico a breve e a lungo termine, questo perche' capiremmo i meccanismi del danno progressivo e permanente. Infatti, la strada verso un vaccino autorizzato e' troppo lunga per aspettare pazientemente. Tuttavia, alla fine, la vaccinazione dovrebbe essere lo scopo ultimo della ricerca su cCMV perche' eviterebbe la trasmissione verticale durante la gravidanza oppure eviterebbe lo sviluppo della malattia nel bambino. Infine, l'identificazione dei biomarker sopracitati potrebbe supportare la futura ricerca sullo svillupo del vaccino.

Per concludere, dalla ricerca presentata in questa tesi, sono emerse diverse questioni che dovrebbero essere tenute in considerazione nella futura ricerca. La maggior parte dei bambini infetti con cCMV sono asintomatici alla nascita ed hanno una buona prognosi per uno sviluppo normale, pertanto e' essenziale la comparazione del loro stato immunologico con quella dei bambini sintomatici alla nascita che hanno un esito peggiore a lungo termine, cosi' come con quelli asintomatici che sviluppano gli stessi LTI, per poter determinare cosa stia succedendo nei bambini con cCMV. Contemporaneamente dovremmo anche definire nuove strategie cliniche e studiare se le attuali strategie cliniche siano vantaggiose per quei neonati asintomatici che sviluppano gli stessi LTI, e se per questo gruppo i benefici, per esempio della terapia antivirale, non superino gli effetti collaterali. Tuttavia, un traguardo ancora piu'urgente dovrebbe essere la standardizzazione delle definizioni di malattia sintomatica e sviluppo di LTI senza la quale non si possono trarre conclusioni affidabili circa la patogenesi di cCMV.

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Roberta Rovito, Hans-Jörg Warnatz, Szymon M. Kiełbasa, Hailiang Mei, Vyacheslav Amstislavskiy, Ramon Arens, Marie-Laure Yaspo, Hans Lehrach, Aloys C.M. Kroes, Jelle J. Goeman* and Ann C.T.M Vossen*. * Shared last authorship. **Impact of Congenital Cytomegalovirus infection on transcriptomes from archived dried blood spots in relation to long-term clinical outcome.** *PLoS One. 2018 Jul 19;13(7):e0200652.*

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CV

Roberta Rovito was born on the 8th of January 1988 in Turin, Italy. In 2007 she graduated from high school at the Istituto Liceo Scientifico Nicolo' Copernico in Turin. She then studied Medical Biotechnology at the Molecular Biotechnology Center from the University of Turin, where she graduated as B.Sc. in 2010. She obtained her M.Sc. degree in Medical Biotechnology in 2012 from the Medicine and Surgery University of Turin. During the Bachelor and Master she undertook an internship in the Molecular Virology Laboratory of the San Luigi Gonzaga Hospital Orbassano in Turin, under the supervision of Professor David Lembo. The goal of such internship was to find antivirals against several viruses such as HSV-1, HSV-2 and HCMV, and evaluate their mode of action. This research led to a scientific publication.

In 2013 she got awarded with a Marie-Curie scholarship and became a fellow in VacTrain (Training network for the next generation vaccinologists), a research network funded by the 7th Framewotk People Programme of the European Commission. In this context, she pursued her PhD studies at Leiden University Medical Center, The Netherlands, in the Department of Medical Microbiology under the supervision of Dr. A.C.T.M. Vossen. The aim of the project was to find prognostic markers and correlates of protection in the congenital Cytomegalovirus infection. The results of her research are published in various scientific journals and are included in this thesis.